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13. ABSTRACT (Maximum 200 Words)
Neuronal and astrocyte cell cultures from the cerebellum and fibroblasts and epithelial cells form the skin and kidney (respectively) of DNA repair mutant mice were examined for the acute and delayed toxicity to nitrogen mustard (HN2) and the related alkylating agent methylazoxymethanol (MAM). Cerebellar neurons from DNA repair mutant mice (i.e., $Mgmt^{-/-}$) Were more sensitive to HN2 and MAM than comparably treated wild type, $Aag^{-/-}$ or $Xpa^{-/-}$ neurons. Cerebellar neurons from $Mgmt^+$ mice were partially protected from the acute toxicity of MAM, but not HN2. A similar pattern of sensitivity was observed for long-term HN2- and MAM-treated cerebellar neurons or fibroblasts an epithelial cells form DNA repair-deficient (i.e., $Mgmt^{-/-}$, $Aag^{-/-}$) mice, the loss of cerebellar neurons, degeneration and the integrity of dopaminergic neurons were especially severe in the brains of $Mgmt^{-/-}$ mice administered MAM. In vivo studies with HN2 also revealed a significant loss of wild type cerebellar neurons that was essentially blocked in comparably treated DNA repair-deficient mice (i.e., $Aag^{-/-}$, $Xpa^{-/-}$). These findings are consistent with our hypothesis that HN2 and MAM selectively target neural and non-neural cells via a DNA damage mechanism.

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INTRODUCTION

Experiments were proposed to examine the molecular mechanism by which mustard chemical warfare agents induce neuronal cell death. DNA damage is the proposed underlying mechanism of mustard-induced neuronal cell death. We proposed a novel research strategy to test this hypothesis by using mice with perturbed DNA repair to explore the relationship between mustard-induced DNA damage and neuronal cell death. Initial *in vitro* studies (Years 1-3) were proposed to examine the cytotoxic and DNA damaging properties of the sulfur mustard analogue mechlorethamine (nitrogen mustard or HN2) and the neurotoxic DNA-damaging agent methylazoxymethanol (MAM) using neuronal and astrocyte cell cultures from different brain regions of mice with perturbed DNA repair. Findings from these studies were used to examine the *in vivo* neurotoxic effects of HN2 and MAM (Years 3-5) in mice with perturbed DNA repair.

BODY OF THE REPORT
STATEMENT OF WORK FOR YEARS 1-5 of FUNDING (Year 5 was Extension Period)

Year 1 (9/01/1998-9/01/1999):
1. Obtain *Aag-* and *Mgmt*-deficient mice from L. Samson and establish colonies.
2. Expand current colony (2 breeder pairs) of ERCC1-deficient mice.
3. Develop neuronal and astrocyte cell cultures from wild type mice.
4. Examine cytotoxicity of HN2 and MAM in wild type neuronal and astrocyte cell cultures.
5. Develop neuronal and astrocyte cultures from DNA repair-deficient mice.

Year 2 (9/01/1999 – 9/01/2000):
1. Examine wild type and DNA repair-deficient neuronal and astrocyte cell cultures treated with HN2 or MAM-induced cytotoxicity.
2. Measure DNA damage in wild type neuronal and astrocyte cell cultures for HN2 & MAM.
3. Commence dose-range finding studies of HN2 and MAM in wild type mice.

Year 3 (9/01/2000 – 9/01/2001):
1. Continue measuring DNA repair-deficient neuronal and astrocyte cell cultures treated with HN2 or MAM-induced cytotoxicity.
2. Measure DNA damage in DNA repair-deficient neuronal and astrocyte cell cultures for HN2 & MAM.
3. Commence dose-range finding studies of HN2 and MAM in DNA repair mutant mice.

Year 4 (9/01/2001 – 9/01/2002):
1. Complete DNA damage analysis of MAM and HN2 in DNA repair-deficient neuronal and astrocyte cell cultures
2. Examine HN2 and MAM treated DNA repair mutant mice for neuropathology.

Year 5 (9/01/2002 – 9/01/2003):
1. Complete DNA damage analysis of HN2 & MAM in DNA repair mutant neuronal & astrocyte cell cultures.
2. Finish analyzing HN2 treated repair mutant mice for neuropathology.

OVERVIEW

The overall goal of studies proposed during Years 1-4 were to: (i) compare the vulnerability of neuronal and astrocyte cell cultures to HN2 and MAM in both wild type and DNA repair mutant mice (Years 1-3), (ii) examine the DNA damaging properties of HN2 and MAM in neuronal cell cultures of wild type and DNA repair-mutant mice (Years 2-4) and (iii) examine the *in vivo* neurotoxic effects of MAM (Years 3 and 4) and HN2 (Year 5) in wild type and DNA repair mutant mice. A detailed description of the research accomplishments for each objective in Years 1-5 follows.

YEAR 1

Animals

For these studies, we breed four strains of mice that either overexpress (1 strain) or are deficient (3-strains) in three key proteins of different DNA repair pathways. These are *ERCC1, Mgmt* (1-overexpression, 1-knock-out), and *Aag*. The primary objective of Year 1 studies was to establish animal colonies for each of the four different mouse mutants and one wild type strain (i.e., C57BL/6J) (Objectives 1 & 2) and to develop neuronal and astrocyte cell cultures from each of these mouse strains (Objective 3 & 5). All four of these objectives were completed during Year 1. Animal colonies were developed from all mouse mutants and the wild type strain, except for the *ERCC1* knock-out mouse. Unfortunately, male and female *ERCC1* mice were found to be infertile and, thus, they were replaced with another mouse mutant strain, the *Xpa* knock-out mouse. Like *ERCC1*, the *Xpa* null mutant mouse is nucleotide excision-repair (NER) deficient, but loss of this particular repair protein results in the inability to recognize certain DNA lesions (e.g., bulky DNA adducts, x-links) [26,43]. Therefore, *Xpa* knock-out mice would be a particularly useful animal model for determining if HN2-induced x-links play an important role in mustard-induced neuronal and non-neuronal cell death [22,39]. Unfortunately, we found out later through extensive breeding that null X null mutants have reduced fertility requiring aggressive expansion of this colony to obtain sufficient animals for subsequent experiments. This mouse mutant strain was used throughout the rest of the grant period.

Development of Cell Cultures

The brains of wild type and DNA repair mutant mice were used to develop primary neuronal and astrocyte cell cultures. Unfortunately, the generation of $Xpa^{-/-}$ and $Aag^{-/-}$ was a particular problem in Year 1 (and throughout the grant period) with only 1 litter generated every 2 months (*average/yr*). Therefore, efforts were established early on to expand each of these colonies in order to meet the objectives of the grant. This required the use of wild type mice in Year 1 to generate heterozygous mouse mutants for obtaining both *Aag* and *Xpa* null mice. Consequently, studies in Year 1 were focused on developing only neuronal cultures from both wild type and DNA repair mutant mice. [As it turns out, this was an excellent strategy because this cell type was found in Year 2 to be relatively insensitive to both MAM and HN2.] This became a more significant problem in both Years 3 and 4, which required a large number of young animals to complete both the dose-range finding studies and the subsequent *in vivo* neurotoxicity studies for MAM and HN2. Therefore, one of our chief objectives in Year 1 (and throughout the grant) was to increase the number of breedings to complete the objectives planned for both the neuronal cell cultures and whole animal studies.

Cytotoxicity Studies

One of the objectives of Year 1 studies was to develop neuronal and astrocyte cell cultures from different brain regions (i.e., cortex, midbrain, cerebellum) of wild type mice and examine these cultures for sensitivity to HN2 and MAM. Because of the problems encountered with the generation of DNA repair-deficient mice colonies and our previous extensive experience with rat cerebellar neuronal cultures, these studies were limited to the development of mouse cerebellar neuronal cultures. [The development of murine cortical and midbrain cultures were postponed until Year 2]. Neuronal cultures were developed from the cerebellum of $Mgmt^{-/-}$, $Xpa^{-/-}$, $Xpa^{+/-}$ and $Mgmt^{+}$ mice and examined for their sensitivity to HN2, MAM or UV irradiation. The limited number of these animals prevented us from extensive study of the comparative sensitivity to HN2 or MAM and thus, the results were <u>preliminary</u>. Neuronal cells (i.e., $Xpa^{-/-}$ & $Xpa^{+/-}$) were UV irradiated because this produces DNA lesions that are repaired by nucleotide excision repair, the same pathway that repairs cross-links induced by HN2.

Mgmt$^+$ cerebellar neurons indicate they are protected from MAM, but not HN2-induced cytotoxicity. [This is consistent with more comprehensive studies of these mouse mutants treated with MAM and HN2 in Year 2]. In separate experiments, we demonstrated that *Xpa*$^{-/-}$ cerebellar neurons are more sensitive than *Xpa*$^{+/-}$ neurons to both HN2 and low-dose UV irradiation [8]. These results are consistent with previous studies that demonstrated that *Xpa*$^{-/-}$ and *Xpa*$^{+/-}$ cerebellar neurons are sensitive to UV irradiation. Cerebellar neurons from both *Xpa*$^{-/-}$ and *Xpa*$^{+/-}$ mice have been previously shown to respond in a similar manner to UV irradiation. Though preliminary (only 1 experiment with n=4), these findings did at least suggest that the survival of cerebellar neurons depends on the repair of HN2- and MAM-induced DNA damage. Additional studies in Year 2 were conducted to determine if these effects are consistent and differ among cell types (i.e., neurons vs. astrocytes).

YEAR 2

Animals

In Year 2, we encountered significant difficulties in maintaining all three strains of DNA repair mouse mutants as a result of the untimely death of one of our technicians, Mr. Greg Komma, who had sole responsibility for the mouse room. Although several technicians helped to maintain our wild-type stocks, they were not trained to take over this responsibility on a moment's notice and we recognized eventually that animals had been identified incorrectly as bearing *Mgmt* and *Aag* deficiencies were used for additional breedings. Therefore, rather than to try to recover these strains in house, we eliminated these strains and re-derived them again from breeder pairs sent to us in July from Dr. Leona Samson (Harvard University, Boston, MA). These events were a serious setback for the studies planned in Year 2 and unfortunately this necessitated the postponing of many experiments until Year 3. To prevent further disruptions of the animal colony, measures were taken to train two additional technicians to be fully proficient in all aspects of animal breeding and genotyping.

Development of Cell Cultures

One of the objectives of the studies in Year 2 was to develop neuronal and astrocyte cell cultures from different brain regions (i.e., cortex, midbrain, cerebellum) of wild type and DNA repair-deficient mice and examine them for sensitivity to HN2 and MAM. Because of the shortage of DNA repair mutant mice (i.e., *Mgmt*, *Aag*) and the fact that timed-pregnant mutant mice (GD14-15) would have to be sacrificed, the studies in Year 2 were limited to the use of wild type, *Mgmt*$^+$, and *Xpa*$^{-/-}$ mice. Cerebellar neuronal and astrocyte cell cultures were developed from both wild type and *Mgmt*$^+$ 6-7 day old postnatal mice while cortical and midbrain neuronal cultures were developed from wild type (i.e., C57BL/6) mouse embryos (GD14-15). Using the same litter of animals, we were able to develop separate and viable (80-90%) neuronal cell cultures from the cortex and midbrain of mouse embryos (GD14-15). Unlike the cerebellar neuronal cultures, the cortical and midbrain cultures were technically challenging because 2-3 litters of mouse embryos were required to generate enough cells for one experiment. Moreover, yields of neuronal cells were substantially lower for embryonic tissue (~0.54 to 0.23 x 10^6 cells/fetal brain) compared to postnatal tissue (1.5-2.0 x10^6 cells/neonatal brain). Midbrain neuronal cultures were immunoprobed with antibodies to glutamate decarboxylase (GAD) and tyrosine hydroxylase (TH) to determine the density of GABAergic and dopaminergic neurons. A majority of the neurons (>90%) were observed to be GABAergic, while only a small fraction (~1%) were dopaminergic.

Cytotoxicity Studies

The primary objective of the 2nd year of study was to generate a sufficient number of postnatal and fetal DNA repair-deficient mice to assess the cytotoxicity of HN2 and MAM in cerebellar or cortical and

midbrain neuronal cell cultures (respectively). While we experienced difficulty in generating *Aag-* and *Mgmt*-deficient mice, we were successful in determining the relative sensitivity of HN2 and MAM using *Mgmt*⁺ and *Xpa*⁻/⁻ mice. The objective of these studies was to determine if there were regional and cell type differences among brain regions of wild type and DNA repair mutant mice for MAM and HN2-induced cytotoxicity. Initial studies compared the relative toxicity of the sulfur mustard analogue mechlorethamine (HN2, nitrogen mustard) and the neurotoxin methylazoxymethanol (MAM) among cerebellar neuronal and astrocyte cell cultures prepared from mice that overexpress human O^6-methylguanine-DNA alkyltransferase (MGMT⁺) or are deficient in the nucleotide excision repair protein xeroderma pigmentosum group A (XPA). Extensive cell loss was observed in wild-type mouse cerebellar neurons treated with 1000 μM MAM (>75%) ($p<0.001$) and >5.0 μM HN2 (>30%)($p<0.001$) (*see* **Fig. 1A**, Appendix). In contrast, photomicrographs of similarly treated *Mgmt*⁺ cerebellar neurons demonstrated that neurons that overexpress the human DNA repair protein (which was determined by western blotting) protected these cells from MAM, but not from HN2-induced cytotoxicity. The increased resistance of *Mgmt*⁺ neurons to high concentrations of MAM may be explained by the elevated repair of O^6-methylguanine DNA adducts, a lesion that may be responsible for MAM-induced neuronal cell death. [This hypothesis is confirmed by the results from more comprehensive studies of the *in vitro* and *in vivo* cytotoxic properties of MAM in *Mgmt*⁻/⁻ and *Mgmt*⁺ mice in Years 3 and 4.] *Mgmt*⁺ neurons were not protected from HN2-induced cytotoxicity because this genotoxin produces predominantly N^7-alkyl DNA adducts and x-links, DNA lesions not repaired by MGMT [28]. In comparison to mouse cerebellar neurons, wild type or MGMT⁺ cerebellar astrocytes were relatively insensitive to 100 μM MAM or 1.0 μM HN2-induced cytotoxicity. [This appears to be a consistent feature of astrocytes whether they are from wild type or DNA repair mutant mice as determined later on in Years 3-5]. Although preliminary, these studies indicate that DNA repair plays a very important role in protecting neurons from MAM.

In comparison to cerebellar neurons from *Mgmt*⁺, neuronal survival after exposure to 50 μM – 500 μM MAM for 24 h was similar for wild-type and *Xpa*⁻/⁻ cells (*see* **Fig. 1B**, Appendix). In contrast, *Xpa*⁻/⁻ neurons were more sensitive to HN2 than wild type cells ($p<0.001$). The increased sensitivity of NER-deficient neurons to HN2 and UV-irradiation (Year 1 studies) and not MAM suggests that the inefficient removal of x-links plays an important role in mustard-induced neurotoxicity.

We also examined the sensitivity of neurons from the cerebral cortex (*see* **Fig. 1C**, Appendix) and midbrain (*see* **Fig. 1D**, Appendix) of fetal mice treatment with either MAM or HN2. As discussed above, the limited number of cells from these two brain regions only allowed us to examine a small range of MAM (0-500 μM) and HN2 (0.1 to 10 μM) concentrations (n=4, 1 experiment). In comparison to cerebellar neurons, neurons from the cerebral cortex and midbrain appeared to be less sensitive to MAM or HN2-induced toxicity. [These studies were continued in the early part of Year 3 and included a more comprehensive range of both MAM and HN2 for both cortical and midbrain neuronal cultures].

DNA Damage Studies
Because our colony of wild type mice were being used to expand the three DNA repair-deficient mutant mice (i.e., *Xpa, Mgmt, Aag*), a sufficient number of animals were not available in Year 2 to develop high density neuronal cell cultures (1-2 x 10⁷ cells/flask or dish) that are required for the DNA damage studies. These studies were postponed until Year 3 when we expected that we would have sufficient numbers of animals in the colonies to begin the DNA damage studies.

Dose-Range Finding Studies
Because of the difficulties experienced with the generation of DNA repair-deficient mice for the preparation of neuronal and astrocyte cell cultures, dose-range finding studies proposed in Year 2 were postponed until Year 3.

YEAR 3

Animals
Despite significant problems encountered in the first two years of the grant, we were able to get back on 'track' and complete most of the objectives proposed in Year 3. This success was attributed to an increased breeding strategy and the efficient genotyping of mice by two highly trained technicians, which provided a sufficient number of animals to meet a majority of the proposed objectives. Despite these aggressive efforts (2 neuronal and/or astrocyte cell cultures/every other week), we were still unable to generate a sufficient number of animals to complete both the acute (24h) and sub-chronic (up to 7d) cytotoxicity and DNA damage studies. Moreover, skin fibroblast cell lines were established for each strain to clarify the acute (24h) neurotoxic properties of HN2 and MAM. Consequently, studies in Year 3 focused on examining the <u>acute</u> cytotoxic and DNA damage properties of HN2 and MAM in neurons, astrocytes, and fibroblast cell cultures. The sub-chronic toxicity studies were postponed until Year 4.

Development of Cell Cultures
Neuronal and astrocyte cell cultures from the cerebellum of wild type and DNA repair-deficient mice (i.e., $Mgmt^{-/-}$, $Aag^{-/-}$, $Xpa^{-/-}$) were developed to compare their sensitivity to HN2 and MAM. Skin fibroblast cell cultures were also developed from each strain (i.e., $Mgmt^{-/-}$, $Aag^{-/-}$, $Xpa^{-/-}$) because these cells are prime targets for mustards [34] and, therefore, the sensitivity of these cells to mustards may occur by a similar mechanism. Although not originally proposed, similarities in the sensitivity of neuronal and skin fibroblasts to HN2 or MAM would also suggest that non-neuronal cells could be used as a good predictor of HN2- or MAM-induced neurotoxicity.

Cytotoxicity Studies
The selective vulnerability of neurons within the CNS is one of the key features of Parkinson's disease and related neurodegenerative disorders. Consistent with this notion, we demonstrate that wild type cerebellar neurons (*vs.* astrocytes) are selectively vulnerable to MAM and HN2 (**Fig. 2A and B**) and that this vulnerability differs, at least *in vitro*, on a regional level (cerebellar > cortex > midbrain) for mature neurons (**Fig. 2C and 2D**). We show that cerebellar neurons are more sensitivity to MAM and HN2 (especially at high concentrations) than comparably treated cortical or midbrain neurons. [This was confirmed by whole animal studies in Years 4 & 5 with MAM demonstrating that cerebellar neurons are more vulnerable than neurons from other brain regions]. However, cerebellar neurons were also more sensitive to MAM than HN2, at least at the concentrations tested (*compare* **Figs. 2C and 2D**). These studies demonstrated that wild type cerebellar neurons were (i) more sensitive to HN2 and MAM than neurons from other brain regions (i.e., cortex, midbrain), (ii) more sensitive to HN2 and MAM than cerebellar astrocytes, and (iii) the sensitivity of HN2 and MAM treated wild type neurons correlated with an increase in markers of apoptosis (i.e., nuclear changes, mitochondrial membrane permeability) and DNA damage (i.e. strand breaks) (*see* **Fig. 4**). Since cerebellar neurons were determined to be the most sensitive CNS cell type to the acute toxic effects of MAM and HN2, cerebellar neuronal (granule cell) cultures were used throughout the remainder of the *in vitro* toxicity studies with DNA repair mutant mice in Years 3-5. More importantly, MAM specifically targets cerebellar granule cells when the genotoxicant is administered to young rats or mice [4] and ectopic and multinucleated granule cells have

been observed in the cerebellum of subjects with Guam ALS/PDC [33,37]. Therefore, cerebellar granule cells are an excellent neuronal cell type to compare the *in vitro* and *in vivo* neurotoxic and DNA damaging properties of MAM and HN2 and their potential role in acute or chronic brain injury.

The mechanism underlying this regional and cell specific vulnerability was further examined by comparing the acute neurotoxicity of HN2 and MAM in cerebellar neuronal cultures derived from mice with deficits in three different cellular DNA repair pathways [i.e., direct reversal (MGMT), base-excision (AAG) and nucleotide excision (XPA)] (*see* **Fig. 3**). The direct reversal pathway primarily repairs O^6-methylguanine DNA lesions formed by alkylating agents while the base-excision and nucleotide excision repair pathway repair N7-methylguanine (and oxidative) or cross-links (and bulky adducts). Consequently, if specific DNA lesions are responsible for MAM or HN2-induced neurotoxicity then we would expect to see differences in the sensitivity of neurons from each of the DNA repair mutants to these genotoxicants. To test this hypothesis, we treated cerebellar neuronal cell cultures derived form $Aag^{-/-}$, $Mgmt^{-/-}$ and $Xpa^{-/-}$ mice for 24h or up to 7days to examine the acute and sub-chronic toxic effects of MAM and HN2. Because of the limited number of animals for these studies, the sub-chronic studies were postponed until Year 4. Studies in Year 3 demonstrated that DNA repair-proficient ($Aag^{+/+}$, $Mgmt^{+/+}$, $Xpa^{+/+}$) neurons are sensitive to MAM and HN2, but neurons that lack DNA repair ($Aag^{-/-}$, $Mgmt^{-/-}$, $Xpa^{-/-}$) could either be more sensitive or relatively resistant to these genotoxicants. Particularly noteworthy was the remarkable difference in the sensitivity of $Aag^{-/-}$, $Mgmt^{-/-}$ and $Xpa^{-/-}$ neurons to either MAM or HN2. In year 3, we demonstrated that $Aag^{-/-}$ neurons were relatively resistant to the acute neurotoxic effects of HN2 or MAM than comparably treated wild type (C57BL/6J) cells. Although unexpected, these findings are consistent with the resistance of bone marrow cells from $Aag^{-/-}$ mice to the acute cytotoxic effects of alkylating agents [29]. The basis for this protection is reported to be an imbalance in enzymes of the base-excision DNA repair pathway [25] and this may explain why $Aag^{-/-}$ neurons appeared insensitive to MAM or HN2. However, this hypothesis may be an oversimplification because MAM and HN2 generate different types of DNA lesions and HN2-induced DNA lesions (i.e, x-links or alkylguanyl adduct) are not known to be substrates for $Aag^{-/-}$. Moreover, recent studies indicate that many DNA repair enzymes or proteins have multiple roles within cells and it is possible that 'knocking out' DNA repair may also influence these other cellular processes. Later on, we also show in in vivo studies and cDNA microarrays (Year 5) that MAM and HN2 also induce different mechanisms of *in vivo* cell death.

In contrast to $Aag^{-/-}$ neurons, $Mgmt^{-/-}$ neurons were more sensitive to MAM or HN2 than comparably treated wild type cells. An important point from these studies is that the O^6-methylguanine DNA adduct, which is produced at much lower levels (~1-6%) than the corresponding N7-methylguanine DNA lesion (~70-80%), appears to be the key lesion that is responsible for the acute and long-term neurotoxic effects of MAM. Overall, $Mgmt^{-/-}$ cerebellar neurons appeared to be very sensitive to the acute toxic effects of both MAM and HN2. These findings suggest that O^6-methylguanine DNA adducts appear to play an important role in the acute neurotoxicity of MAM. However, the increased sensitivity of $Mgmt^{-/-}$ neurons to HN2 was unexpected since this genotoxicant is not known to produce an O^6-alkylguanine DNA lesion [28]. The increased sensitivity of $Mgmt^{-/-}$ cells to HN2 appears to be an important cellular mechanism of protection since *Mgmt*-deficient skin fibroblasts (*see* **Fig. 5**) and kidney epithelial cells (*see* **Fig. 9,** Year 4) also share a similar sensitivity to HN2. Taken together, the findings from Year 3 studies indicate that DNA repair capacity/DNA damage is an important determinant of the vulnerability of neurons to the acute toxic effects of both HN2 and MAM.

The mechanism of the increased sensitivity of cerebellar neurons to MAM and HN2 was further investigated by examining toxin treated neurons for markers of apoptosis, a suicide cellular process for the removal of damaged or injured cells. Apoptosis is an active, genetically regulated cellular mechanism that involves nuclear alterations (chromatin condensation followed by fragmentation), loss of mitochondrial membrane permeability that lead to the destruction of damaged cells. The metabolic pathways involved in apoptosis have been elucidated and a variety of well-established techniques are used to examine cells for early, active and late events. Using a combination of these techniques, wild type neurons treated with MAM or HN2 were examined for well-known markers of different periods of apoptosis, that is, a loss in the mitochondrial membrane potential psi (an early event) and DNA fragmentation (TUNEL labelling; a late-stage event). Because of the shortage of DNA repair-deficient mice, these studies were limited to examining markers of apoptosis in MAM and HN2 treated cerebellar neuronal cell cultures from wild type mice (i.e., C57BL/6). Wild type cerebellar neurons were treated with MAM and HN2 for 24h and examined for mitochondrial membrane potential (DePsipher™, Trevigen, Inc) (**Fig. 4A,** Appendix) and DNA fragmentation (NeuroTacs™, Trevigen, Inc) (**Fig. 4B,** Appendix). The mitochondrial membrane potential (delta psi) of wild type cerebellar neurons was preserved (*punctate orange-red aggregates*) at most MAM concentrations, but was severely perturbed (*diffuse cytoplasmic green fluoresence*) at high concentrations of MAM (>100 μM). In contrast, low concentrations of HN2 (>1.0 μM) induced significant loss of the mitochondrial membrane potential suggesting that the mechanism of neuronal death induced by HN2 differs from that induced by MAM. This was substantiated by recent microarray analysis of cerebellar neuronal cell cultures treated with low doses of MAM (100 μM) or HN2 (1.0 μM), which demonstrated little overlap (<20%) between the network of genes that were activated by MAM or HN2 (**see Fig. 23, Year 5**). These differences were particularly striking when comparing the effect of MAM on the cerebellum of different DNA repair mutant backgrounds such as $Mgmt^+$ [*compare* **Figs. 11 and 13**, Year 4]. Consistent with these findings, DNA fragmentation (**Fig. 4B,** Appendix) was significantly elevated (*p<0.001 or p <0.01*, respectively) only in cerebellar neurons treated with high concentrations of MAM (1000 μM) or HN2 (5.0 μM and 10 μM).

As discussed above, we also examined the sensitivity of DNA repair-deficient skin fibroblasts to MAM and HN2 (*see* **Fig. 5,** Appendix). We demonstrate that fibroblast cell cultures, like neuronal cultures developed from DNA repair mutant mice, exhibited a differential sensitivity to MAM and HN2 that paralleled the sensitivity of neurons to these genotoxicants. In short-term studies (24h), $Mgmt^{-/-}$ and $Xpa^{-/-}$ skin fibroblasts appeared particularly sensitive to MAM and HN2. A comparison of the short-term (i.e., acute) and long-term (i.e., delayed) survival of HN2 and MAM-treated non-neuronal tissue (i.e., fibroblasts) in Year 3 studies has provided evidence to support our hypothesis that DNA damage is an important mechanism underlying the delayed neurotoxicity of these alkylating agents.

DNA Damage Studies
Because our colony of wild type mice were being used to expand the three DNA repair-deficient mutant mice (i.e., *Xpa, Mgmt, Aag*), a sufficient number of animals were not available in Year 2 to develop high density neuronal cell cultures (1-2 x 10^7 cells/flask or dish) that are required for the DNA damage studies. These studies were postponed until Year 4 when we expected that we would have sufficient numbers of animals in the colonies to begin the DNA damage studies.

Dose-Range Finding Studies
Because of the difficulties experienced with the generation of sufficient DNA repair-deficient mice for the preparation of neuronal and astrocyte cell cultures for the cytotoxicity and DNA damage studies,

dose-range finding studies proposed in Year 2 and Year 3 were postponed until Year 4. In the meantime, dose-range findings studies were initiated in wild type mice by treating 3 day old pups with a high dose of MAM (30 mg/kg, s.c.) to establish methods for assessing neuronal cell loss, neurodegeneration and DNA damage in MAM and HN2 treated DNA repair mice. The dose and treatment age were chosen based upon several important factors: (i) the cerebellum (notably the granule cell layer) is severely compromised in mouse pups treated at 1-5 days with MAM, (ii) we demonstrated that cultures of cerebellar neurons of wild type and $Mgmt^{-/-}$ were especially sensitive to HN2,(see above studies) and (iii) $Mgmt^{+}$ partially protects cerebellar granule cell cultures from MAM-induced neurotoxicity. Two litters of C57Bl6 mice (n=8/litter) were administered either a single subcutaneous dose of MAM (30 mg/kg, n=4/litter) in saline or a similar volume of saline (control). The animals were examined daily for changes in body weight and size (length from crown to rump), features that typically are reduced in MAM treated animals. [A comprehensive analysis of the growth of wild type and DNA repair mutant mice after MAM and HN2 treatment is shown in **Fig. 10**, Year 4]. As previously reported, the body weight of MAM treated animals was 20-25% lower than saline treated littermates and remained lower up until termination at day 21. Animals were perfused with 4% buffered paraformaldehyde and the brain and spinal cord cryoprotected in sucrose. Coronal brain tissue sections were made through the whole cerebellum, the serial sections stored at $-90°C$ in cryoprotectant, and every tenth section examined for cerebellar morphology (cresyl violet or anti-calbindin-D staining), neurodegeneration (Fluoro-Jade B or silver staining). Calbindin-D is an intracellular calcium-binding protein that is especially abundant in Purkinje cells of the cerebellum and is, thus, a very useful marker for Purkinje cell degeneration. Fluoro-Jade B is a polyanionic fluorescein derivative useful for the histological staining of neurons undergoing neurodegeneration [30] while components of neurons undergoing degeneration (e.g., lysosomes, axons, terminals) become agryrophilic (affinity for silver ions) and upon reduction form dark grains that are visible by light microscopy. Gross observation of the cerebellum from MAM treated mice revealed extensive atrophy of the cerebellar lobes (stars) when compared with the cerebellum of saline treated mice (**Fig. 6A**, Appendix). This was more evident in cresyl violet stained (**Fig. 6B**, Appendix) and anti-calbindin immunoprobed (**Fig. 6C**, Appendix) coronal sections of the cerebellum from MAM treated animals. These stains revealed extensive hypogranulation of the cerebellum and the disorganization and displacement of neurons within both the granule and Purkinje cell layers. The disorganization of granule and Purkinje cells was also evident in coronal sections stained with the nuclear stain DAPI or Fluoro-Jade B (**Fig. 6D**), a high affinity fluorescent probe that has been used to detect degenerating neurons in rodents administered excitotoxins [31]. Particularly noticeable was the heavy deposition of silver stain (arrows) over neurons within the molecular layer of the cerebellum (**Fig. 6E**) an indication that these cells are injured or damaged.

These studies demonstrate that MAM severely disturbs the cytoarchitecture of the young cerebellum by inducing severe neuronal loss of cerebellar granule cells and neurodegeneration via a DNA damage mechanism. Our studies are consistent with a recent report indicating that MAM-induced cell death of the external granule cell layer of the cerebellum of wild type mice has recently been observed in 3-day old postnatal wild type mice administered an intraperitoneal injection of MAM [10]. More comprehensive studies are reported in Year 4 by examining the extent of cerebellar neuronal loss, neurodegeneration and DNA damage in both MAM treated wild type and DNA repair deficient mice. Comparable studies of HN2 treated wild type and DNA repair mutant mice are reported in Year 5.

YEAR 4

Animals

The generation of $Xpa^{-/-}$ mice appeared to be a particular problem with only 1 litter generated every 2 months during Years 3 to 5. [A comprehensive list of all of the wild type and DNA repair mutant animals used for the *in vivo* studies can be found in **Table I**, Year 4]. Therefore, *in vivo* neurotoxicity studies proposed in Year 4 were limited to the use of wild type, $Mgmt^{-/-}$, $Aag^{-/-}$ and $Mgmt^{+}$ mice. Kidney epithelial cell cultures (like skin fibroblasts) developed from each strain (i.e., $Mgmt^{-/-}$, $Aag^{-/-}$ and $Xpa^{-/-}$) were used to clarify the delayed (*cloning efficiency*) neurotoxic properties of HN2 and MAM. The balance of the animals (both wild type and DNA repair mutant) were used for dose-range finding studies and to examine for neuropathological changes. However, we were still unable to generate a sufficient number of animals to complete both the MAM and HN2 *in vivo* neurotoxicity studies. Consequently, we applied for a no-cost extension and it was granted (9/02/02) to complete the *in vivo* neurotoxicity studies with HN2. Therefore, wild type and the three DNA repair mutant mice were used primarily in Year 4 studies to: (i) complete the *in vitro* acute toxicity studies of HN2 and MAM, (ii) examine the *in vitro* delayed neurotoxic properties of HN2 and MAM and (iii) examine the *in vivo* neurotoxic properties of HN2 and MAM. A detailed description of the research accomplishments for each objective of Year 4 follows. Despite significant problems encountered in the first three years of the grant with our animal colonies (breeding, re-deriving each line) and personnel (death of a technician), we were able to complete most of the objectives proposed in Year 4.

Development of Cell Cultures

The above studies from Years 1-3 demonstrate that the targeted reduction of DNA repair (i.e., $Mgmt^{-/-}$, $Xpa^{-/-}$) within neurons or skin fibroblasts increases their sensitivity to HN2 or MAM, possibly *via* the production of specific DNA adducts (e.g., cross-links, O^6-methylguanine). In Year 3 studies, we demonstrated that fibroblast cultures developed from DNA repair mutant mice exhibited a differential sensitivity to MAM and HN2. In short-term and long-term studies, $Mgmt^{-/-}$ and $Xpa^{-/-}$ fibroblasts appeared particularly sensitive to MAM and HN2. Similar studies were conducted in Year 4 with kidney epithelial cells derived from wild type or DNA repair deficient (i.e., $Aag^{-/-}$, $Mgmt^{-/-}$, and $Xpa^{-/-}$) mice. The objective of these studies was to determine if the increased sensitivity of DNA repair-deficient skin fibroblasts to MAM and HN2 is also shared by other non-neuronal cell types (i.e., kidney epithelial cells).

Cytotoxicity Studies

The central hypothesis under study is that DNA damage is a primary mechanism of mustard-induced and MAM-induced neuronal cell death. In support of this hypothesis, we demonstrated in Years 1-3 that neurons that are deficient in $Mgmt^{-/-}$ appear to be particularly vulnerable to the acute toxic effects of MAM or HN2. The increased sensitivity of $Mgmt^{-/-}$ neurons to HN2 is, however, less clear since mustards are not known to produce DNA adducts that are repaired by MGMT. However, recent studies suggest there is considerable cross-talk among DNA repair pathways [11,25] and this could account for the increased sensitivity of $Mgmt^{-/-}$ neurons to HN2. Therefore, if MGMT$^{-/-}$ neurons are indeed sensitive to both HN2 and MAM, then we would expect that neurons that overexpress MGMT ($Mgmt^{+}$) should be protected from the acute toxicity of both of these genotoxins. To test this hypothesis, $Mgmt^{+}$ and $Mgmt^{-/-}$ cerebellar neurons (*see* **Fig. 7,** Appendix) were treated with similar concentrations of HN2 and MAM and examined for cell viability by measuring mitochondrial function (Alamar Blue™) and the live/dead assay (calcein AM/propidium iodide). In comparison to $Mgmt^{-/-}$ or wild type neurons, significant protection was only observed at high concentrations of MAM (1000 μM) while survival was essentially similar for wild type and $Mgmt^{+}$ neurons at all concentrations of HN2 tested. These findings suggest

that O^6-methylguanine DNA adducts plays a more important role in the acute neurotoxicity of MAM than HN2, but MGMT only provided partial protection. This is also consistent with the reduced viability of $Mgmt^{-/-}$ neurons to lower concentrations of MAM for longer treatment times (see Fig. 9, Appendix). $Mgmt^+$ mice were not available for long-term studies with MAM, but we would predict (*based upon the findings from the Mgmt$^{-/-}$ experiments*) an increased protection from the long-term neurotoxic effects of MAM. The inability of MAM to induce significant neuropathological changes in the cerebellum of 22 day-old $Mgmt^+$ mice after a single injection of the genotoxicant on postnatal day 3 (*see* **Fig. 13**, Year 4) is additional data that supports this hypothesis.

The increased sensitivity of $Mgmt^{-/-}$ neurons to HN2 is, however, less clear since the DNA lesions produced by HN2 (i.e.,7-alkylguanine, x-links) are not known to be substrates for MGMT [28]. In fact, this is the reason why the $Mgmt^{-/-}$ mouse mutant was included in the grant as a negative control. These unexpected findings (Year 4) suggested that maybe one or both of the HN2-induced DNA lesions were responsible for the increased sensitivity.

The notion that the persistence of DNA adducts may be associated with delayed neurotoxicity is supported by the increased sensitivity of fibroblasts (*see* **Fig. 5**, Appendix) from $Mgmt^{-/-}$ and $Xpa^{-/-}$ after a brief (24h) exposure to MAM or HN2. The insufficient number of $Xpa^{-/-}$ mice for these experiments limited the delayed studies to wild type, $Aag^{-/-}$ and $Mgmt^{-/-}$ mice. Therefore, additional studies were conducted with wild type, $Aag^{-/-}$, and $Mgmt^{-/-}$ mice to determine if the increased sensitivity of $Mgmt^{-/-}$ neurons to HN2 and MAM also occurs after prolonged exposure (*up to 7 days*) to the genotoxins (*see* **Fig. 8**, Appendix). Compared to wild type and $Aag^{-/-}$ neurons, $Mgmt^{-/-}$ neurons were more sensitive to low concentrations of HN2 (>1.0 μM) and MAM (>100 μM) and cell loss increased with time. These studies demonstrate that high concentrations of mustards and MAM are acutely toxic to neurons while low concentrations induce a delayed neurotoxicity. The unexpected sensitivity of $Mgmt^{-/-}$ neurons to HN2 suggests that mustards either produce O^6-methylguanine DNA adducts or cellular pathways that repair HN2-induced cross-links (e.g., NER, recombination, mismatch) are also perturbed in $Mgmt^{-/-}$ neurons. However, gene expression profiling of MAM and HN2 treated cerebellar neurons using cDNA microarrays that were performed during Year 5 (*see* **Fig. 23**, Appendix) demonstrated that several NER, recombination, and mismatch repair proteins were not altered by MAM or HN2. Therefore, additional studies are underway using microarrays and proteomics to determine if other repair pathways (e.g., MGMT) play an important role in HN2-induced neurotoxicity.

In Year 3 studies, we demonstrated that fibroblast cultures developed from DNA repair mutant mice exhibited a differential sensitivity to MAM and HN2. In preliminary short-term studies (24h), $Mgmt^{-/-}$ and $Xpa^{-/-}$ fibroblasts appeared particularly sensitive to MAM and HN2. Long-term studies with fibroblasts revealed a similar pattern of vulnerability to HN2 and MAM. Similar studies were conducted in Year 4 with kidney epithelial cells from wild type, $Aag^{-/-}$, $Mgmt^{-/-}$, and $Xpa^{-/-}$ mice to determine if a similar pattern of vulnerability occurs among different tissues treated with HN2 or MAM. Like skin fibroblasts, epithelial cell lines prepared from the kidneys of $Aag^{-/-}$, $Mgmt^{-/-}$, $Xpa^{-/-}$ were examined for their sensitivity to HN2 or MAM (*see* **Fig. 9**, Appendix). A similar pattern of sensitivity emerged for skin fibroblasts and kidney epithelial cells treated with HN2 or MAM for 24h and the cells examined for survival 2-3 weeks later (*cloning efficiency*). Like neurons, skin fibroblasts and kidney epithelial cell cultures from $Aag^{-/-}$ mice were relatively insensitive to HN2 and MAM, while similarly treated cells from $Mgmt^{-/-}$ and $Xpa^{-/-}$ mice were sensitive to both genotoxins. However, $Xpa^{-/-}$ skin fibroblasts appeared more sensitive to HN2 and $Aag^{-/-}$ kidney epithelial cells appeared more sensitive to MAM than comparably treated kidney or fibroblast cell cultures, respectively. A possible explanation for this

differential response is that individual cell types may display complex phenotypic differences with respect to the cellular repair of DNA damage induced by alkylating agents [29]. Therefore, these studies suggest that non-neuronal cells exhibit different sensitivities to HN2 and MAM.

DNA Damage Studies

One of the primary hypotheses under study in this grant is that DNA damage plays an important role in neuronal cell death. Findings from the viability studies in Years 1 and 2 and the above studies demonstrated that $Mgmt^{-/-}$ neurons are especially sensitive to both MAM and HN2 suggesting that O^6-alkylguanine DNA lesions or x-links play a major role in the acute neurotoxic effects of MAM while the exact mechanism of HN2 is unknown. The basis for this increased sensitivity is likely to be an increase in unrepaired damage to the DNA lesions induced by MAM (i.e., N7-methylguanine, O^6-methylguanine) or HN2 (N7-alkylguanine, x-links). Inefficient removal of these DNA lesions would lead to abasic sites or strand breaks and ultimately cell death. To clarify the relationship between DNA damage and the increased sensitivity of $Mgmt^{-/-}$ neurons to these agents, we compared the extent of DNA damage (i.e., strand breaks) induced by HN2 and MAM in wild type, $Aag^{-/-}$, $Mgmt^{-/-}$, and $Mgmt^+$ neurons. To test this hypothesis, cerebellar neurons from wild type and DNA repair mutant ($Aag^{-/-}$, $Mgmt^{-/-}$, and $Mgmt^+$) mice were plated at the same density (140-150K cells/well), treated for 24h with the same concentrations of MAM (10 μM, 100 μM, 1000 μM) or HN2 (1.0 μM, 5.0 μM, 10 μM) and the paraformaldehyde fixed cells examined for DNA damage using the NeuroTacs™ kit (Trevigen, Inc.), which detects DNA strand breaks by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique. In general, DNA damage increased with the concentration of either HN2 or MAM among all four genotypes. Cell counts were only available for MAM and HN2 treated wild type, $Mgmt^{-/-}$ and $Mgmt^+$ cerebellar neurons (*see* **Fig. 7B**, Appendix) and the results are consistent with the viability studies (*see* **Fig. 7A**, Appendix). As expected, DNA damage was significantly higher in $Mgmt^{-/-}$ neurons treated with MAM than comparably treated wild type ($p < 0.01$) or $Mgmt^+$ ($p < 0.001$) neurons. The pronounced protective effect of MGMT on MAM-induced DNA damage and neuronal viability, and later on in MAM-induced cerebellar degeneration (*see* **Fig. 14**, Appendix), is strong evidence in favor of the formation of O^6-methylguanine DNA adducts (*vs*. N^7-methylguanine DNA adducts) in MAM-induced neurotoxicity. Unlike MAM, MGMT did not protect neurons against HN2-induced DNA damage even though viability studies indicate that cell survival was significantly higher in $Mgmt^+$ neurons when compared to similarly treated $Mgmt^{-/-}$ or wild type neurons. However, close examination of the previous viability studies indicates that MGMT only provided partial protection since substantial (~50%) cell loss was observed at high concentrations of HN2. In contrast, the viability of MAM treated $Mgmt^+$ neurons was ~3x higher than comparably treated $Mgmt^{-/-}$ neurons. These findings would suggest that HN2 (*vs*. MAM) may induce cell death by multiple pathways (e.g., alkylation-induced DNA damage, oxidative stress) and MGMT only provided partial protection from the genotoxin. Additional studies are underway to clarify the mechanism of HN2-induced neurotoxicity by examining the role of other DNA repair pathways (e.g., recombination, oxidative) or antioxidant enzymes (e.g., GSH). However, GSH levels in HN2 treated wild type or $Mgmt^{-/-}$ cerebellar neurons was unremarkable (*see* **Fig. 16**, Appendix) suggesting that the increased sensitivity of $Mgmt^{-/-}$ neurons to HN2 is probably not mediated by an oxidative stress mechanism. Analysis of DNA from HN2 treated $Mgmt^{-/-}$ neurons (currently underway) for different DNA lesions (e.g., O^6-alkylguanine, GMOH, x-links) will provide insight into the mechanism underlying mustard-induced neurotoxicity.

Dose-Range Finding Studies

Evidence from the acute and delayed toxicity studies of neuronal cell cultures with $Mgmt^{-/-}$, $Aag^{-/-}$, and $Mgmt^+$ mice conducted in Years 1-3 and the studies above suggested that we should begin dose-range

findings studies with these DNA repair mouse mutants. *In vivo* studies conducted in Year 3 demonstrated that 3 day-old mice injected subcutaneously with 30 mg/kg MAM and examined 3 weeks later for viability (only two deaths) only developed moderate neuronal cell loss and neurodegeneration in the cerebellum (*see* **Fig. 6**). At this dose of MAM, the body weight and size (length from crown to rump) of wild type mice was also reduced, as previously reported [1]. Based upon these preliminary findings, dose-range finding studies in Year 4 were conducted in wild type mice by treating 3 day-old pups with three different doses of MAM: a high (43 mg/kg), moderate (21.5 mg/kg) or low dose (4.3 mg/kg) and the results compared with similarly treated DNA repair mutant (*Mgmt$^{-/-}$*, *Aag$^{-/-}$* and *Mgmt^{+}*) mice. For comparison, wild type and DNA repair mutant mice were also injected with HN2 (high: 40 mg/kg, 20 mg/kg; moderate: 10 mg/kg, 5 mg/kg; and low: 2.6 mg/kg) in saline or a similar volume of saline (control). All animals were examined for viability (*see* **Table I** and **Fig. 10**, Appendix) and neuropathology after a single subcutaneous dose of MAM (high, moderate, low doses) (*see* **Figs. 11-14**, Appendix) or HN2 (high, moderate, low doses) (*see* **Figs. 17-20**, Year 5, Appendix). The neuropathological examination of MAM treated mice was conducted in Year 4 while similar studies of HN2 treated mice was performed in Year 5. Animals were examined daily for changes in body weight and size and periodically observed (*weekly*) for signs of motor dysfunction (hindlimb splay, ataxia, lethargy), features that are typically induced by MAM in rodents. As previously reported in Year 3 studies, the body weight of MAM (i.e., 43 mg/kg and 21.5 mg/kg) treated animals was typically 20-25% lower than saline treated littermates for both wild type and DNA repair-deficient mice (i.e., *Mgmt$^{-/-}$*, *Aag$^{-/-}$*) and remained lower up until termination at day 22 (*see* **Fig. 10**). Similar results were obtained for wild type and DNA repair deficient mice (i.e., *Aag$^{-/-}$*) treated with HN2 (10 mg/kg, 5 mg/kg, 2.6 mg/kg). Body weights for the low MAM or HN2 dosed animals were similar to saline treated animals. HN2 was particularly toxic to both wild type and *Aag$^{-/-}$* mice at doses > 5 mg/kg with 100% of *Aag$^{-/-}$* mice dying within 3 days of dosing and 50% of *Aag$^{-/-}$* mice dying (LD$_{50}$) at 2.5 mg/kg. However, 100% of wild type and *Aag$^{-/-}$* mice lived at lower concentrations of HN2 (1.3 mg/kg) or MAM (4.3 mg/kg). *Mgmt$^{-/-}$* and *Mgmt^{+}* mice were only dosed with MAM and LD50 determined to be 21.5 mg/kg for *Mgmt$^{-/-}$* mice while none of the MAM treated *Mgmt^{+}* mice died before termination (22 days). The LD50 for MAM in wild type mice was determined to be 43 mg/kg while 100% of the *Mgmt$^{-/-}$* mice died at this dose of the genotoxin. These studies indicate that wild type, *Aag$^{-/-}$* and *Mgmt$^{-/-}$* mice are very sensitive to high concentrations of HN2 and MAM with a majority (>50%) of the animals living either at moderate or low concentrations of the genotoxins. Results from these dose-range findings studies are consistent with our previously reported sensitivity of *Mgmt$^{-/-}$* neurons to MAM or, conversely, the increased protection of *Mgmt^{+}* neurons from MAM-induced toxicity. The increased sensitivity of *Aag$^{-/-}$* mice to high concentrations of MAM may be explained by the increased sensitivity of β-islets to alkylating agents [3]. Results from the HN2 studies are described in Year 5.

Animals were perfused with 4% buffered paraformaldehyde and the brain and spinal cord cryoprotected in sucrose. Sagittal brain tissue sections were made through the whole cerebellum, the serial sections stored at −90°C in cryoprotectant, and every tenth section examined for cerebellar morphology (cresyl violet [A] or anti-calbindin-D [B] staining), neurodegeneration (silver staining, [C]) or dopaminergic neurons (i.e., anti-tyrosine hydroxylase, [D]). Calbindin-D is an intracellular calcium-binding protein that is especially abundant in Purkinje cells of the cerebellum and is, thus, a very useful marker for Purkinje cell degeneration. Components of neurons undergoing degeneration (e.g., lysosomes, axons, terminals) become agryrophilic (affinity for silver ions) and upon reduction form dark grains that are visible by light microscopy. Cresyl violet stained sections of the cerebellum from MAM treated wild type mice revealed extensive atrophy of the cerebellar lobes (*stars*) when compared with the cerebellum of saline treated mice (*see* **Fig. 11A**, Appendix). This was more evident in cresyl violet stained (*see* **Fig.**

13A, Appendix) and anti-calbindin immunoprobed (*see* **Fig. 13B**, Appendix) sagittal sections of the cerebellum from MAM treated *Mgmt$^{-/-}$* mice. These stains revealed extensive hypogranulation of the cerebellum and the disorganization and displacement of neurons within both the granule (GL) and Purkinje (PL) cell layers. Particularly noticeable was the heavy deposition of silver stain (*arrows*) over neurons within the molecular layer of the cerebellum (*see* **Fig. 13C**, *Inset*) of *Mgmt$^{-/-}$* mice, an indication that these cells are injured or damaged. MAM was also observed to have additional effects on the midbrain (i.e., substantia nigra, SN) of treated mice, a brain region not known to be affected by MAM. Tyrosine hydroxylase immunoreactivity was noted to be reduced in SN neurons (*arrows*) of wild type mice treated with MAM (*see* **Fig. 11D**, Appendix), but this effect was more evident in *Mgmt$^{-/-}$* mice treated with a 2-fold lower dose of MAM (*see* **Fig. 13D**, Appendix). Comparable studies with either *Aag$^{-/-}$* mice (*see* **Fig. 12**, Appendix) or *Mgmt$^+$* mice (*see* **Fig. 14**, Appendix) demonstrated that the cerebellum and midbrain from these animals were less perturbed by MAM. These latter findings with *Aag$^{-/-}$* and *Mgmt$^+$* mice are consistent with the previously reported protection of neuronal cultures derived from these mice treated with MAM.

These *in vivo* findings with MAM in *Mgmt$^{-/-}$*, *Aag$^{-/-}$*, and *Mgmt$^+$* mice are consistent with the findings from our previous *in vitro* studies with the same mutant mice. Therefore, we provide strong *in vitro* and *in vivo* evidence that MAM induces its neurotoxic effects *via* the generation of O^6-methylguanine DNA adducts, data which supports our original hypothesis. Comparable studies were conducted with *Mgmt$^{-/-}$*, *Aag$^{-/-}$*, and *Mgmt$^+$* mice administered HN2 to determine if the extent of cerebellar loss, neurodegeneration and DNA damage differs (or is similar) to that of MAM treated DNA repair mutant mice. These studies were our primary focus in the no-cost extension period.

YEAR 5

Animals
Wild type and DNA repair mutant (*Aag$^{-/-}$*, *Mgmt$^{-/-}$* and *Xpa$^{-/-}$*) mice were used primarily in the no-cost extension period (Year 5) to: (i) complete the *in vitro* acute toxicity studies of HN2 and MAM, (ii) examine the *in vivo* neurotoxic properties of HN2 and (iii) identify the molecular mechanisms of HN2 and MAM-induced neurotoxicity. Although, we had sufficient wild type mice to conduct the *in vivo* studies proposed in Years 3-5, the generation of DNA repair mutant mice was a particular problem (**see Table I**, Year 4, Appendix). Therefore, one of our objectives in the 1-yr extension was to increase the number of breedings to complete the objectives of both the neuronal cell cultures and whole animal studies. This provided us with a sufficient amount of mutant mice to finish the *in vitro* and *in vivo* cytotoxicity studies, but not enough to identify the specific DNA lesions responsible for the increased sensitivity. This would have required twice the number of animals to obtain sufficient amount of DNA for these studies, which was cost-prohibitive. The balance of the animals (both wild type and DNA repair mutant) were used for dose-range finding studies and to examine for neuropathological and neurobehavioral changes. Despite these problems, we were able to complete most of the objectives proposed in Year 5. Moreover, cDNA microarrays were used during the 1-year extension period to begin identifying the molecular targets that may be responsible for the increased sensitivity of DNA repair-mutant neurons to HN2 and MAM. [A manuscript describing the results from these studies has been recently submitted to *PNAS* for publication].

Cytotoxicity Studies
In Years 3 and 4, we demonstrated that *Mgmt$^{-/-}$* neurons were particularly sensitivity to both MAM and HN2. An important point from the MAM studies is that the O^6-methylguanine DNA adduct, which is

produced at much lower levels (~1-6%) than the corresponding N7-methylguanine DNA lesion (~70-80%), appears to be the key lesion that is responsible for the acute and long-term neurotoxic effects of MAM. The objective of experiments in Year 5 was to determine if the increased sensitivity of $Mgmt^{-/-}$ neurons to HN2 is due to the formation of cross-links or N7-alkylguanine DNA lesions. To test this hypothesis, we examined compared the sensitivity of all three DNA repair mutants to HN2 and its monofunctional structural analogue 2-chloroethylamine (CEA). Unlike HN2, CEA does not form cross-links with DNA and, therefore, is significantly less genotoxic and mutagenic than HN2 [42]. Therefore, CEA would predominantly alkylate nitrogen atoms in DNA of neurons to produce monoadducts (e.g., N^7-alkylpurines) and not cross-links (N7-alkyl-alkyl-N7) [41]. The objective of these experiments was to determine if the increased sensitivity of $Mgmt^{-/-}$ neurons to HN2 is due to the formation of cross-links or N7-alkylguanine DNA lesions. Another benefit of these studies is they would provide important critically important information about the role of specific DNA lesions in HN2-induced neurotoxicity. We compared the sensitivity of wild type (C57BL/6), $Aag^{-/-}$, $Mgmt^{-/-}$, and $Xpa^{-/-}$ cerebellar neurons to mechlorethamine (HN2) and 2-chloroethylamine (CEA) (*see* **Fig. 15**, Appendix). Mouse cerebellar neurons were treated with similar concentrations (1.0 μM to 10 μM) of HN2 or CEA and cell survival determined 24h later by the live/dead assay (*for details see* **Fig. 1**). Wild type and $Aag^{-/-}$ neurons were equally sensitive to HN2 than CEA. These studies suggest that AAG or the base-excision repair pathway is probably not involved in the repair of either mustard-induced N7-alkylguanine DNA lesions or x-links. However, significant differences were noted in the sensitivity of $Mgmt^{-/-}$ and $Xpa^{-/-}$ neurons to HN2 or CEA. $Mgmt^{-/-}$ neurons were significantly more sensitive ($p < 0.001$) to HN2 than CEA. In contrast, $Xpa^{-/-}$ neurons were more sensitive to CEA than HN2. These studies suggest that N7-guanyl monoadducts produced by CEA are repaired primarily by the NER pathway while MGMT is involved in the repair of HN2-induced x-links or an O^6-alkylguanine DNA lesion. Plosky *et al.* [27] have recently demonstrated that embryonic fibroblasts from $Xpa^{-/-}$ mice are especially inefficient at removing N7-alkylguanine DNA lesions from active genes. Comparable studies with $Aag^{-/-}$ mouse embryo fibroblasts revealed little removal of N7-alkylguanine DNA lesions from active genes. These recent findings are consistent with the increased sensitivity of $Xpa^{-/-}$ neurons to CEA and a similar sensitivity of $Aag^{-/-}$ and wild type neurons to CEA.

An alternative explanation for the increased sensitivity of $Mgmt^{-/-}$ neurons to HN2 is that this mustard, like sulfur mustard [19], may also react with the O^6-position of guanine to generate the unstable chloroalkylguanine lesion, which under physiological conditions would generate the more stable hydroxyalkylguanine DNA lesion [19]. In support of this hypothesis, Dr. Ludeman's group at Duke University have recently demonstrated that reducing cellular MGMT levels with O^6-benzylguanine sensitizes chinese hamster ovary (CHO) cells to HN2 [2]. Using authentic O^6-alkylguanine DNA lesion of HN2 synthesized by Dr. Ludeman, we have recently detected the formation of this O^6-alkylguanine DNA lesion calf thymus DNA incubated with micromolar concentrations of HN2. These recent studies strongly suggest that HN2 can form low levels of this DNA lesion when purifed DNA is incubated with HN2. Studies are now underway to determine if HN2 treated $Mgmt^{-/-}$ cells (e.g., skin fibroblasts, kidney epithelial cells) have elevated levels of these DNA lesions.

The above viability studies with HN2 and CEA demonstrate that $Mgmt^{-/-}$ and $Xpa^{-/-}$ neurons are particularly sensitive to these genotoxic agents, possibly by the generation of cross-links or N7-alkylguanine DNA adducts, respectively. However, the influence of MAM and HN2 on mitochondrial function (Year 3&4) and their reported generation of reactive oxygen species [14] or influence on cellular antioxidant enzymes (e.g., GSH) [21] suggests that the increased sensitivity of these cells to HN2 and MAM may be occur through oxidative stress. To test this hypothesis, we compared the

sensitivity of cerebellar neurons from wild type, $Aag^{-/-}$, $Mgmt^{-/-}$ and $Xpa^{-/-}$ mice after exposure to HN2 and CEA or MAM. Mouse cerebellar neurons from wild type, $Aag^{-/-}$, $Mgmt^{-/-}$ and $Xpa^{-/-}$ mice were treated with various concentrations (1.0 μM to 20 μM) of HN2 or CEA (0.1 μM to 10 μM) and cellular GSH levels determined 24h later using the GSH specific fluorophore monochlorobimane (MCB) (*see* **Fig. 16**, Appendix). MCB is a lipophilic and GSH specific probe that passively diffuses across cellular membranes into the cytoplasm and has been previously used to measure intracellular levels of GSH in primary rat glial and neuronal cell cultures [5,40]. In general, these studies indicate that the effect of CEA or HN2 on neuronal GSH levels differed and was dependent upon the genotype. In wild type cells, intracellular GSH levels were similar after HN2 or CEA treatment, except high concentrations of CEA reduced GSH levels. GSH levels were also reduced by high concentrations of HN2 (>5.0 μM) in $Xpa^{-/-}$, neurons, but the levels remained unchanged in neurons from other DNA repair mutant mice (i.e., $Aag^{-/-}$ or $Mgmt^{-/-}$) were essentially similar to wild type cells. Particularly interesting was the induction of GSH levels in MGMT$^{-/-}$ neurons after CEA, but not HN2 treatment. This would suggest that the increased sensitivity of $Mgmt^{-/-}$ neurons to HN2 is not mediated by altered GSH levels, but rather by a DNA damage mechanism. The exact DNA lesions (i.e., O^6-alkylguanine, GMOH, or x-links) responsible for the increased sensitivity of $Mgmt^{-/-}$ cells to HN2 are likely to be revealed following the analysis of DNA from mustard treated $Mgmt^{-/-}$ cells.

MAM is an established alkylating agent and, unlike HN2, is not known to alter intracellular GSH levels. In parallel with the HN2 studies described above, we also examined MAM treated neuronal cultures from wild type and DNA repair mutant mice (i.e., $Aag^{-/-}$, $Mgmt^{-/-}$, or $Xpa^{-/-}$) for intracellular GSH levels. In general, MAM did not appreciably influence intracellular GSH levels in either wild type or DNA repair mutant neurons. However, GSH levels were significantly higher in $Mgmt^{-/-}$ neuronal cultures treated with MAM (>200 μM), the repair mutant most sensitive to MAM. The reason for this selective increase in GSH for $Mgmt^{-/-}$ neurons is unknown, but may be an alternative mechanism to explain the incomplete protection of MGMT$^+$ neurons or brain tissue from MAM-induced neurotoxicity. These studies suggest that the increased sensitivity of DNA repair mutant neurons to MAM or HN2 are not primarily mediated through an oxidative stress mechanism.

DNA damage Studies
Although a sufficient number of mutant mice were available during Year 5 to finish the *in vitro* and *in vivo* cytotoxicity studies, it did not provide enough animals during the grant period (Years 2-4) to identify the specific DNA lesions responsible for the increased sensitivity. This would have required twice the number of animals to obtain a sufficient amount of DNA to complete these studies (~50 μg DNA/sample for HN2 studies; ~20 μg sample for MAM studies), which was limited by both the amount of available animal housing and funds. Recoveries from a typical cerebellar granule cell culture were ~5 μg/1x10^6 cells (~1 mouse brain) requiring pooling of samples for HPLC/EC or LC/MS. Consequently, we have been collecting samples over the extension period (*using other resources*) from neuronal and non-neuronal cell cultures of wild type and DNA repair mutant mice treated with either genotoxin to finish the DNA lesion studies (i.e., MAM:O^6-methylguanine, N7-methylguanine; HN2: GMOH, x-links). Our objective is to pool the samples so that we will have a sufficient amount of DNA to analyze by HPLC/EC or LC/MS. We have also recently established a collaboration (10/03) with Dr. D. Doerge (NCTR, Jefferson, AK) to analyze our DNA samples for MAM-induced DNA lesions using the very sensitive technique of LC/MS/MS. Dr. Doerge has kindly agreed to analyze the MAM samples (at no cost) for O^6-methylguanine. The rest of the samples will be analyzed by HPLC/EC for HN2-induced (i.e., GMOH) or MAM-induced (i.e., N7-methylguanine) DNA lesions. Sample collection and analysis should be completed by August 2004.

Dose-Range Finding Studies

Additional studies were conducted in Year 5 to continue examining wild type and DNA repair mutant (*Mgmt*$^{-/-}$, *Aag*$^{-/-}$, and *Mgmt*$^{+}$) mice for viability and neuropathology (*see* **Figs. 17-20**) after a single subcutaneous dose of HN2 (high: 40 mg/kg, 20 mg/kg, 10 mg/kg; moderate: 5 mg/kg and 2.6 mg/kg; low: 1.3 mg/kg) in saline or a similar volume of saline (control). Similar results were obtained for wild type and DNA repair deficient mice (i.e., *Aag*$^{-/-}$) treated with HN2 (10 mg/kg, 5 mg/kg, 2.6 mg/kg). Body weights for the low MAM or HN2 dosed animals were similar to saline treated animals. HN2 was particularly toxic to both wild type and AAG$^{-/-}$ mice at doses > 10 mg/kg with 100% of the animals dying within 3 days of dosing and 50% of the animals dying (LD$_{50}$) at 5 mg/kg. However, 100% of wild type and AAG$^{-/-}$ mice lived at lower concentrations of HN2 (2.6 mg/kg, 1.3 mg/kg) or MAM (4.3 mg/kg).

These studies indicate that wild type, *Aag*$^{-/-}$ and *Mgmt*$^{-/-}$ mice are very sensitive to high concentrations of HN2 and MAM with a majority (>50%) of the animals living either at moderate or low concentrations of the genotoxins. Results from these dose-range findings studies are consistent with our previously reported sensitivity of *Mgmt*$^{-/-}$ neurons to MAM or, conversely, the increased protection of *Mgmt*$^{+}$ neurons from MAM-induced toxicity. The increased sensitivity of AAG$^{-/-}$ mice to high concentrations of MAM may be explained by the increased sensitivity of β-islets to alkylating agents [3]. Results from the HN2 studies are inconclusive at this time until additional studies are conducted (*no-cost extension*) with wild type and DNA repair mutant mice.

Dose-Range Finding Studies

Persistent DNA damage, either from excessive alkylation and/or reduced DNA repair, is a possible mechanism by which HN2 or MAM could have persistent effects on CNS function. Previous studies demonstrate that MAM disturbs neuronal development within the cerebellum of 1-5 day old neonatal mice [1,13]. Our studies in Year 3 and 4 are consistent with these findings by demonstrating that the postnatal cerebellum of wild type mice is sensitive to MAM (*see* **Figs 6 and 11**). Wild type (C57BL/6), *Mgmt*$^{-/-}$ and *Mgmt*$^{+}$ 3-day old neonatal (PND3) mice were injected with saline or MAM and, 24h later, the brain sectioned, stained and examined by light microscopy for neuropathology. Studies conducted in Year 5 demonstrate that wild type, *Aag*$^{-/-}$ and *Mgmt*$^{-/-}$ mice are very sensitive to high concentrations of HN2 while a majority (>50%) of the mice lived either at moderate or low concentrations of the genotoxins. The sensitivity of these DNA repair mutant mice to MAM was associated with pronounced changes in the cerebellum. Results from the MAM dose-range findings studies are consistent with the increased sensitivity of the cerebellum from *Mgmt*$^{-/-}$ mice. Studies in Year 5, continued examining wild type and DNA repair mutant (*Mgmt*$^{-/-}$, *Aag*$^{-/-}$, and *Mgmt*$^{+}$) mice for viability and neuropathology after a single subcutaneous dose of HN2 (high: 40 mg/kg, 20 mg/kg, 10 mg/kg; moderate: 5 mg/kg and 2.6 mg/kg; low: 1.3 mg/kg) in saline or a similar volume of saline (control). Animals were examined daily for changes in body weight, size and periodically observed (*2-3 times/wk*) for signs of motor dysfunction (hindlimb splay, ataxia, lethargy), features that typically occur in MAM treated animals. As previously reported, the body weight of MAM (i.e., 43 mg/kg and 21.5 mg/kg) treated animals were typically 20-25% lower than saline treated littermates for both wild type and DNA repair deficient mice (i.e., MGMT$^{-/-}$, AAG$^{-/-}$) and remained lower up until termination at day 22 (**see Fig. 10**). Similar results were obtained for wild type and DNA repair-deficient mice (i.e., *Aag*$^{-/-}$) treated with HN2 (10 mg/kg, 5 mg/kg, 2.6 mg/kg). Body weights for the low MAM or HN2 dosed animals were similar to saline treated animals. HN2 was particularly toxic to both wild type and *Aag*$^{-/-}$ mice at doses > 10 mg/kg with 100% of the animals dying within 3 days of dosing and 50% of the animals dying (LD$_{50}$) at 5 mg/kg.

However, 100% of wild type and $Aag^{-/-}$ mice lived at lower concentrations of HN2 (2.6 mg/kg, 1.3 mg/kg) or MAM (4.3 mg/kg) (see **Table I**, Appendix). $Mgmt^{-/-}$ and $Mgmt^+$ mice were only dosed with MAM and LD50 determined to be 21.5 mg/kg for $Mgmt^{-/-}$ mice while none of the MAM treated $Mgmt^+$ mice died before termination (22 days). The LD50 for MAM in wild type mice was determined to be ~43 mg/kg while 100% of the $Mgmt^+$ mice died at this dose of the genotoxin. These studies indicate that wild type, $AAG^{-/-}$ and $MGMT^{-/-}$ mice are very sensitive to high concentrations of HN2 or MAM while a majority (>50%) of the mice lived either at moderate or low concentrations of the genotoxins. Results from these dose-range findings studies are consistent with the increased sensitivity of $Mgmt^{-/-}$ cerebellar neuronal cultures to HN2 or MAM. The increased sensitivity of $Aag^{-/-}$ mice to high concentrations of MAM may be explained by the increased sensitivity of these mice to alkylating agents [29].

Persistent DNA damage, either from excessive alkylation and/or reduced DNA repair, is a possible mechanism by which HN2 or MAM could have persistent effects on CNS function. Previous studies demonstrate that MAM disturbs neuronal development within the cerebellum of 3 day old (PND3) neonatal mice [1,13]. In vivo studies in Year 4 demonstrated that overepxressing MGMT and a lack of AAG protects cerebellar neurons from MAM-induced cell death and that cultures of cerebellar neurons from these DNA repair mutants exhibited a similar sensitivity to MAM or HN2. Since the in vitro studies (see **Fig. 15**, Appendix) in Year 5 also demonstrate that MGMT protects cerebellar neurons from HN2-induced cell death, additional studies were conducted to determine if this DNA repair protein also protects in vivo developing cerebellar neurons from HN2.

Wild type (C57BL/6), $Mgmt^{-/-}$, $Aag^{-/-}$ and $Xpa^{-/-}$ 3-day old neonatal (PND3) mice were administered a single injection of saline or HN2 (5 mg/kg, s.c.) and, 24h (wild & $Mgmt^{-/-}$) or 22 days ($Aag^{-/-}$ & $Xpa^{-/-}$) later, the brain sectioned, stained and examined by light microscopy for neuropathology. Because of the large loss of DNA repair mutant mice after HN2 injection (see Section 5), tissue blocks were only available from PND 4 wild type and $Mgmt^{-/-}$ mice while PND22 tissue was only available from $Xpa^{-/-}$ and $Aag^{-/-}$. $Mgmt^{-/-}$ mice (total of 20 mice injected with either MAM or HN2 in Year 5) were a particular problem because most of the mice died (HN2: 3/6; MAM: 6/9). Animals were perfused with 4% buffered paraformaldehyde and the brain and spinal cord cryoprotected in sucrose. Sagittal brain tissue sections were made through the whole cerebellum, the serial sections stored at –90°C in cryoprotectant, and every tenth section examined for cerebellar morphology (cresyl violet [A] or anti-calbindin-D [B] staining), neurodegeneration (silver staining, [C] or FluoroJade-B™ [D]). Calbindin-D is an intracellular calcium-binding protein that is especially abundant in Purkinje cells of the cerebellum and is, thus, a very useful marker for Purkinje cell degeneration. Components of neurons undergoing degeneration (e.g., lysosomes, axons, terminals) become agryrophilic (affinity for silver ions) and upon reduction form dark grains that are visible by light microscopy. Fluoro-Jade B is an anionic fluorescein derivative useful for the histological staining of neurons undergoing degeneration and has a greater specific affinity for degenerating neurons than viable neurons. The cresyl violet stained cerebellum from HN2 treated wild type mice (5 mg/kg, s.c.) revealed extensive atrophy of the cerebellar lobes and extensive hypogranulation of the cerebellum when compared with the cerebellum of saline treated mice (see **Fig. 17A**, Appendix). In contrast, anti-calbindin immunoprobed (see **Fig. 17B**, Appendix) sagittal sections demonstrated that this dose of HN2, unlike MAM, did not perturb the Purkinje cell layer. These studies suggest that HN2 and MAM may target in vivo neurons differently. Particularly noticeable was the heavy deposition of silver stain (white arrows) over neurons within the external granule cell layer of the cerebellum (see **Fig. 17C**, Appendix) an indication that these cells are injured or damaged. This was confirmed by FluoroJade B staining of alternate tissue sections that showed increased yellow fluorescence (arrows in **D**) over degenerating neurons within the external (EGL) and

internal (IGL) granule cell layer. Note also the thinning of the EGL and IGL as indicated by the double labeling of FluoroJade B stained sections with the nuclear stain Hoescht 33258. Closer examination of the Hoechst stained tissue sections indicated that many neurons of the external granule cell layer (EGL) were severely damaged and displayed morphological features characteristic of apoptosis (e.g., fragmented nuclei, shrunken cells, apoptotic bodies). Comparable studies with $Mgmt^{-/-}$ mice (*see* **Fig. 19**, Appendix) revealed similar changes between saline and HN2 treated PND4 mice (*see* **Fig. 12**, Appendix), but tissue sections from this mouse were poorly fixed and additional tissue from 2 other recently dosed animals were not available for this report. The neuropathology from all other DNA repair mutants were from 22 day old animals. Studies in Year 4 demonstrated that $Aag^{-/-}$ and $Xpa^{-/-}$ mice were particularly insensitive to MAM and in Year 5 also demonstrated that $Aag^{-/-}$ (**Fig. 18**) and $Xpa^{-/-}$ (**Fig. 20**) mice were relatively insensitive to the neurotoxic effects of HN2. This is clearly evident when one compares the overall cytoarchitechture of the cresyl violet stained sections from both saline and HN2 treated $Aag^{-/-}$ (**Fig. 18A**) or $Xpa^{-/-}$ (**Fig. 20A**) mice. These latter findings with $Aag^{-/-}$ and $Xpa^{-/-}$ mice are consistent with the previously reported protection of neuronal cultures derived from these mice treated with HN2 (*see* also **Fig. 3**, Appendix).

These *in vivo* findings with HN2 in $Aag^{-/-}$, $Mgmt^{-/-}$, and $Xpa^{-/-}$ mice, although preliminary, are consistent with the findings from our previous *in vitro* studies with the same mutant mice. Therefore, we provide *in vitro* and *in vivo* evidence that, like MAM, HN2 induces its neurotoxic effects *via* the generation of DNA damage, data that supports our original hypothesis. The in vivo studies with HN2 were our primary focus in the no-cost extension period.

Gene Expression Profiling: In Vivo Studies
Sulfur and nitrogen mustards generate multiple DNA lesions and one or more of these lesions may be responsible for their toxic properties [7,17,18,20,32]. Failure to repair these DNA lesions would be expected to interfere with transcription and translation resulting in perturbed cell function and eventual death via an apoptotic or necrotic mechanism [6,12,23,38]. While much is known about the initial events surrounding mustard-induced DNA damage, there is little known about the intervening molecular events between DNA damage and cell death. In an attempt to identify the molecular events initiated by MAM- or mustard-induced DNA damage, we examined the cerebellum of MAM-treated wild type mice (*see* **Fig. 22**) and HN2 treated cerebellar granule cell neurons for global gene expression by high density (~26,000 genes) mouse cDNA microarrays (*see* **Fig. 23**). One of our objectives was to determine if MAM and HN2 induced a distinct pattern of gene expression *in vivo* or *in vitro* at a concentration that is known to damage DNA. Another was to determine if the action of MAM or HN2 on neurons activated apoptotic genes. Finally, gene expression profiles were generated from cerebral cortex of corresponding mice or neuronal cultures (*from the same preparation*) treated with the related alkylating agent methylazoxymethanol (MAM, this genotoxin generates N^7-methyl- and O^6-methyl-guanine lesions) to determine if neuronal gene expression is distinct for different DNA damaging agents.

Methylazoxymethanol (MAM) is widely used as a developmental neurotoxin and exposure to its glucoside (i.e., cycasin) is associated with the prototypical neurological disorder western Pacific ALS/PDC. However, the mechanism of MAM-induced brain injury is poorly understood. To identify the potential molecular targets of MAM in the developing nervous system, 3 day-old postnatal C57BL/6 mice (PND3) were injected with MAM (43 mg/kg, s.c.) and the cerebellum and cerebral cortex of PND 4, 8, 15 and 22 mice examined for correlation of phenotypic changes (DNA damage and cytoarchitecture) (*see* **Fig. 21**, Appendix) and gene expression differences measured using microarray assays (27,648 genes) (*see* **Fig. 22**, Appendix). To associate phenotypic changes with potential gene

expression profiles, we first determined the influence of MAM on both the cytoarchitechure and the integrity of DNA by examining the changes in the cerebellum of postnatal mice at different stages of development. At the light microscopic level, smaller cerebellar folia, thinner cerebellar cortex and disorganization of neurons in the granule layer (GL) were first noticeable in the cerebellum of PND8 mice after treatment with MAM and these pathological changes became increasingly evident with age (**Fig. 21A**, *right panels*). Consistent with previous rat studies [9], the effect of MAM on the corresponding cerebral cortex or other forebrain areas was minimal (*data not shown*).

MAM specifically targets guanine bases of DNA to generate N7-methylguanine (N7-mGua) or O^6-methylguanine lesions and this DNA damage reportedly leads to strand breaks (i.e., TUNEL labeling) and neuronal cell death *via* an apoptotic mechanism [10]. As shown in **Fig. 21B**, TUNEL labeling was extensive in the external granule cell layer (EGL) of the PND4 cerebellum and low in the whole cerebellum or other brain regions (i.e., cerebral cortex, corpus callosum) confirming that strand breaks are elevated in the developing cerebellum of postnatal mice treated with MAM. In contrast, N7-mGua was elevated in both the cerebellum and cerebral cortex of PND4 mice after MAM administration (**Fig. 21C**). Although not significant, the level of this DNA lesion remained elevated until PND22. The significant elevation of specific forms of DNA damage (i.e., N7-mGua, strand-breaks) detected at PND4, a stage of cerebellar development that did not exhibit observable morphological changes (**Fig. 21A**, *top panels*), implies that DNA damage induced by MAM appears before disruption of the cytoarchitecture of the developing CNS.

Gene expression results revealed: 1. MAM regulates a distinct set of genes in the cerebellum and cortex. 2. Greater than 80% of the genes affected by MAM were developmentally regulated. 3. MAM induced gene expression changes were predominantly early (PND4) in the cerebellum and delayed (PND 8,15) in the cortex. 4. Persistent gene expression changes were observed in both regions. 5. Functional classes of molecules affected by MAM vary by brain region with the proteasome pathway affected in the cerebellum and DNA repair in the cortex. These results clearly demonstrate that MAM-induced brain injury involves a wide range of molecular pathways than previously thought with a majority involved in CNS development. These data provide the basis for further investigating the key regulatory molecules involved in genotoxin-induced brain injury.

Gene Expression Profiling: In Vito Studies
Mature mouse cerebellar neuronal cultures (7 days *in vitro*; 1×10^6 cells/well) were treated with 1.0 μM HN2 or 100 μM MAM for 24h and DNA, RNA and protein isolated using Tri-Reagent™. Since RNA concentrations were low (10-15 μg/well for 6-well plate), two wells were combined and each combined sample (*n=3*) analyzed for gene expression using mouse cDNA microarrays developed from BMAP clones. Image processing, signal quantification and normalization was performed using methods described on our webpage (http://medir.ohsu.edu/~geneview). Summary statistics such as mean, median, range and standard deviation were calculated for all genes. The background adjusted signal intensity was first calculated for each spot. The mean background-adjusted signal intensity over multiple replications per gene and per experiment was then calculated. Finally, natural logarithmic transformation was applied to the adjusted signal intensities. We have applied selective criteria such as greater than two-fold difference and significance value (*p >0.05*) in all experiments, to reduce the dataset, and performed detailed analyses using class clustering.

We first used hierarchical clustering (Euclidean distance measure and centroid linkage) to cluster genes with similar expression levels. Several of these clusters are also specifically enriched with genes of

known function. As shown in **Fig. 23A**, distinct clusters were observed for HN2 and MAM. The number of genes uniquely regulated by each agent and their overlap was shown in **Fig. 23B**. The global expression patterns were further analyzed by functional classes of molecules such as DNA repair, signaling molecules, apoptosis to find correlations among genes and gene-regulatory networks (**Fig. 23C & D**). Gene expression results revealed: 1. Global gene expression was predominantly up-regulated (~50%) by both genotoxins. 2. The number of down-regulated genes was ~3-fold greater for HN2 than MAM. 3. MAM and HN2 regulated a distinct set of genes with minimal overlap (~20%). 4. Only a few genes were observed to be similarly affected by MAM in both neuronal cultures and the PND4 cerebral cortex (2 out of 53) or cerebellum (83 out of 603) of mice. 5. Functional classes of molecules influenced by MAM and HN2 were distinct with MAM having pronounced effects on cell proliferation, DNA repair, immune response, neurogenesis, signal transduction, transcription, transport and the ubiquitin pathway while HN2 primarily influenced apoptosis, hormones, the proteosome, and protein synthesis. The influence of these two genotoxins on the functional classes of these genes also differed. Of the genes on the BMAP chip, none of the DNA repair genes were either up- or down regulated by HN2. This chip included genes that code for proteins from several DNA repair pathways including NER (e.g., XPA, RPA, PCNA, RAD23a, RAD23b, ERCC2, ERCC3) and homologous (e.g., NBS1,BRCA1) and non-homologous recombination (e.g., DNA-PK) repair pathways. Additional genes for direct reversal and base-excision DNA repair (including MGMT and AAG) and DNA damage response (e.g., p53) are available from our current stock of NIA cDNA clones, but were not run in time for this report. Interestingly, the inability of HN2 to induce several NER genes is consistent with findings from the previous viability studies (Year 4) that demonstrated that $Xpa^{-/-}$ neurons were equally sensitive to HN2 as wild type neurons.

These studies demonstrate that MAM induces distinct effects on neuronal gene expression when compared with other related genotoxins. These differences may account for the variable response of the brain or neurons to different types of DNA damaging agents and explain the short- and long-term effects of these agents on the CNS.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that $Aag^{-/-}$ neurons are resistant to the acute toxic effects of MAM or HN2.
- Demonstrated that $Mgmt^{-/-}$ and $Xpa^{-/-}$ neurons are more sensitive than wild type cells to the acute toxic effects of MAM or HN2.
- Demonstrated that $Mgmt^{+}$ neurons are relatively insensitive to MAM and HN2-induced toxicity.
- Demonstrated that the extent of DNA damage differs for HN2 and MAM among DNA repair deficient neurons (i.e., $MGMT^{-/-}$ vs. $MGMT^{+}$)
- Demonstrated that MAM- and HN2-induced neurotoxicity is <u>not</u> due to an oxidative stress mechanism (i.e., GSH).
- Demonstrated that HN2 primarily targets neurons *via* a DNA damage (i.e., cross-links) mechanism.
- Demonstrated that the long-term survival (i.e., cloning efficiency) of MAM and HN2 treated non-neural cells is dependent upon the DNA repair capacity of cells, evidence that supports a common mechanism of MAM- and HN2-induced DNA damage among tissues.
- Demonstrated that *in vivo* MAM induces severe loss and degeneration of DNA repair-deficient cerebellar neurons (i.e., $Mgmt^{-/-}$) and extensive disorganization of the cerebellar cytoarchitecture and this was associated with neurobehavioral deficits (i.e., motor function).
- Demonstrated that MAM-induced cerebellar neuropathology and neurobehavior (i.e., motor function)

is blocked in certain DNA repair-mutant mice (i.e., $Mgmt^+$, $Xpa^{-/-}$).

- Demonstrated that MAM induces severe reduction of tyrosine hydroxylase within dopaminergic neurons of the substantia nigra (SN) and disturbs the organization of SN neurons.
- Demonstrated that *in vivo* HN2 induces severe loss and neurodegeneration of DNA repair-deficient mice (i.e., $Mgmt^{-/-}$) and extensive disorganization of the cerebellar cytoarchitecture and this was associated with neurobehavioral deficits (i.e., motor function).
- Demonstrated that HN2-induced cerebellar neuropathology and neurobehavior (i.e., motor function) is blocked in certain DNA repair-mutant mice (i.e., $Xpa^{-/-}$).
- Demonstrated that the targeting of cerebellar neurons by HN2 and MAM is associated with distinct changes (e.g., functional classification) in gene expression, both *in vivo* and *in vitro*.

REPORTABLE OUTCOMES

1. Kisby, G.E., Wong, V., Olivas, A., Qin, X., Gerson, S.L., Samson, L., Turker, M.S. Neurons of DNA repair mutant mice are selctively vulnerable to DNA damage. Soc Neurosci Abstr 27:#967.4.

2. Y.W., Kow, Imhoff, B., Ingram, D.K., Kisby, G., Kohama, S.G. The influence of caloric restriction (CR) and age on base excision DNA repair (BER) in the rat brain. Soc Neurosci Abstr (*In press*).

3. Kisby, G.E., Sproles, D., Pattee, P., Nagalla, S.R. Gene expression profiling of the developing brain following treatment with methylazoxymethanol (MAM). Soc Neurosci Abstr (*In press*).

4. Kisby, G.E., Pattee, P., Standley, M., Lu, X., O'Malley, J., Lin, B., Muniz, J., Luo, N.L., Beardsley, D..J., Back, S.J., Nagalla, S.R. Global gene expression is perturbed by the genotoxin methylazoxymethanol (MAM) in both the developing cerebral cortex and cerebellum of postnatal mice. Soc Neurosci Abstr (*In press*), 2003.

5. Kisby, G.E., Standley, M., Pattee, P., J. O'Malley, J., Muniz, J., Olivas, A., Nagalla, S.R. Gene expression in methylazoxymethanol (MAM) is distinct when compared with other DNA damaging agents. Soc Neurosci Abstr (*In press*), 2003.

6. Kisby, G.E., Olivas, A., Lesselroth, H., Muniz, J., Samson, L., Gerson, S., Turker, M. *The neurotoxic properties of methylazoxymethanol (MAM) are dependent upon efficient DNA repair.* Findings from these studies will be presented at the International DNA Repair Conference in Bermuda on December 7-13, 2004.

7. Kisby, G.E. *Genotoxins: Tools for Exploring Mechanisms of Brain Injury.* Invited speaker to talk about the role of DNA damage and DNA repair in methylazoxymethanol (MAM) induced brain injury. Pacific Area Northwest Association of Toxicologists (PANWAT) meeting in Bend, OR on Sept 26 and 27, 2003.

8. Kisby, G.E., Lesselroth, H., Olivas, A., Samson, L., Gold, B., Tanaka, K., Turker, M.S. Role of nucleotide- and base-excision repair in genotoxin-induced neuronal cell death. DNA Repair. *In press.*

9. Kisby, G.E., Lesselroth, H., Olivas, A., Wong, V., Samson, L., Gerson, S., Tanka, K., Turker, M. The neurotoxic properties of nitrogen mustard (HN2) depend on efficient DNA repair. *DNA Repair* (*In preparation*).

10. Kisby, G.E., Pattee, P., Standley, M., Lu, X., O'Malley, J., Lin, B., Muniz, J., Luo, N.L., Beardsley, D..J., Back, S.J., Nagalla, S.R. Global gene expression is perturbed by the genotoxin methylazoxymethanol (MAM) in both the developing cerebral cortex and cerebellum of postnatal mice. *PNAS* (*Submitted*).

11. Kisby, G.E., Standley, M., Pattee, P., J. O'Malley, J., Muniz, J., Olivas, A., Nagalla, S.R. Gene expression in methylazoxymethanol (MAM) is distinct when compared with other DNA damaging agents. *Environmental Health Perspectives: Toxicogenomics* (*In preparation*).

12. Kisby, G.E., Lesselroth, H., Olivas, A., Wong, V., Samson, L., Turker, M.S. The DNA repair protein O^6-methylguanine methyltransferase (MGMT) protects neurons from methylazoxymethanol (MAM)-induced cell death. *J. Neuroscience* (*In preparation*).

CONCLUSIONS

A central hypothesis under study in this grant is that the mechanism of neuronal cell death induced by mustards is initiated by DNA damage. Consequently, our primary objective was to use mouse models with perturbations in DNA repair (deficient and overexpressing) to clarify the molecular mechanisms by which mustards induce cell death or neural injury. For comparison, DNA repair mutant mice were also to be treated with methylazoxymethanol (MAM), an environmental agent that is strongly linked with a prototypical neurological disorder with features of Parkinson's disease and dementia. The selective vulnerability of neurons within the CNS is one of the key features of Parkinson's disease and related neurodegenerative disorders. Consistent with this notion, we demonstrate that DNA repair-deficient neurons (i.e., *Mgmt-/-*, *Xpa$^{-/-}$*) are selectively vulnerable to MAM and HN2 and that this vulnerability differs, both *in vitro* and *in vivo*. The mechanism underlying this vulnerability was examined by comparing the acute and delayed neurotoxicity of HN2 and MAM in cerebellar neurons of mice with deficits in different cellular DNA repair pathways [i.e., direct reversal (MGMT), base-excision (AAG) and nucleotide excision (XPA)]. Findings from these studies indicate that DNA repair capacity/DNA damage play a key role in determining the vulnerability of neurons to the acute and delayed toxic effects of both HN2 and MAM. For example, *Aag$^{-/-}$* cerebellar neurons appeared resistant to both MAM and HN2-induced neurotoxicity while other DNA repair deficient neurons (i.e., MGMT$^{-/-}$, XPA$^{-/-}$) exhibited an increased sensitivity to both genotoxins. Additional studies conducted with fibroblast and epithelial cell cultures from DNA repair-deficient mice demonstrated that non-neural tissues also exhibited a similar pattern of sensitivity to MAM and HN2. Consistent with these findings, the long-term survival of *Aag$^{-/-}$* fibroblasts and epithelial cells treated with HN2 or MAM was equal to or better than comparably treated wild type or DNA repair-deficient fibroblasts (i.e., *Mgmt$^{-/-}$*, *Xpa$^{-/-}$*), respectively. DNA damage was examined in HN2 and MAM treated cerebellar neurons from wild type and DNA repair mutant mice to determine if there was a relationship between the extent and type of damage and acute neurotoxicity. Cerebellar neurons from wild type and DNA repair mutant mice were treated with HN2 and MAM and examined for strand breaks (TUNEL). Although specific DNA lesions were not identified, these studies demonstrate that MAM- and HN2-induce DNA damage (i.e., strand breaks) is dependent upon the efficiency of neuronal DNA repair (i.e., MGMT). However, the extent of DNA

damage did not correlate with <u>acute</u> neurotoxic effects of HN2 for $Mgmt^+$ neurons. One possible explanation is that the persistence of DNA adducts in HN2 treated $Mgmt^+$ neurons may result from the perturbation of other cellular DNA repair pathways by overexpressing human MGMT. Despite these findings, the relative insensitivity of both wild type and $Mgmt^{-/-}$ cerebellar neurons to equimolar concentrations of the monofunctional nitrogen mustard 2-chloroethylamine suggests that cross-links are the primary DNA lesion responsible for HN2-induced neurotoxicity. Moreover, these findings are consistent with previous non-neural . Unexpectedly, neural and non-neural cells of $Mgmt^{-/-}$ mice were both especially sensitive to HN2, a DNA repair protein whose primary function is to remove O^6-alkyl DNA adducts [15]. Since cells deficient in recombination repair or mismatch repair are also particularly sensitive to HN2 [15], these cellular pathways may be disrupted by selectively targeting $Mgmt^{-/-}$ in neural or non-neural cells. Additional studies with cells that are deficient in these DNA repair pathways will be required to assess their contribution to HN2-induced neurotoxicity.

The most important finding from the studies proposed in this grant is that the neurotoxic properties of methylazoxymethanol (MAM), an established developmental neurotoxin and etiological candidate for the prototypical neurodegenerative disorder ALS/PDC, are remarkably influenced by DNA repair. Comparative studies with MAM demonstrated that the generation of O^6-methylguanine DNA adducts is the primary event underlying MAM-induced *in vitro* and *in vivo* neurotoxicity. Particularly noteworthy, was the influence of MAM on the metabolism (i.e., tyrosine hydroxylase) (Year 4) and organization of neurons within the SN neurons and the degeneration of cerebellar neurons 3-4 weeks after toxin administration. These findings suggest that MAM can influence the integrity of dopaminergic neurons an effect that may be related to its role in the human neurological disorder western Pacific ALS/PDC. Recent studies (*under a NIEHS Toxicogenomics grant*) indicate that MAM-induced DNA damage (TUNEL labeling and N7-methylguanine DNA lesions) appears within 24h after dosing of young animals and well before neuropathology suggesting that DNA damage is the key event underlying the *in vivo* neurotoxic effects of MAM. The cerebellum of these animals were also examined by gene expression profiling and within 24h of dosing there was a remarkable down-regulation of genes. We also observed a similar effect of MAM on gene expression in cultured cerebellar granule cell neurons (Year 5). Collectively, these studies strongly suggest that MAM-induced O^6-methylguanine DNA adducts are responsible for the significant changes in gene expression and the ensuing neuropathological and neurobehavioral changes. These studies also provide strong evidence that MAM may be acting like a 'slow toxin' [35,36].

A comparison of the short-term (i.e., acute) and long-term (i.e., delayed) survival of HN2 and MAM-treated neuronal or non-neuronal tissue (i.e., fibroblasts, epithelial cells) in Years 1-5 has provided strong evidence to support our hypothesis that DNA damage is an important mechanism underlying the delayed neurotoxicity of these alkylating agents. Studies in Year 5 (no-cost extension) confirmed this hypothesis by demonstrating that ~ 1 month after HN2 treatment there was a considerable loss of cerebellar neurons. Moreover, alternative mechanisms of HN2- and MAM-neuronal cell death (i.e., GSH) were not shown to be consistent with the increased sensitivity of DNA repair mutant neurons to either genotoxicant. Findings from the neuronal studies complemented the long-term survival studies conducted with fibroblasts and epithelial cells treated with HN2 and MAM in Year 4. Findings from the *in vitro* and *in vivo* studies in this grant have provided sufficient evidence to demonstrate that DNA damage is a primary mechanism of both MAM- and HN2-induced neuronal cell death.

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APPENDIX
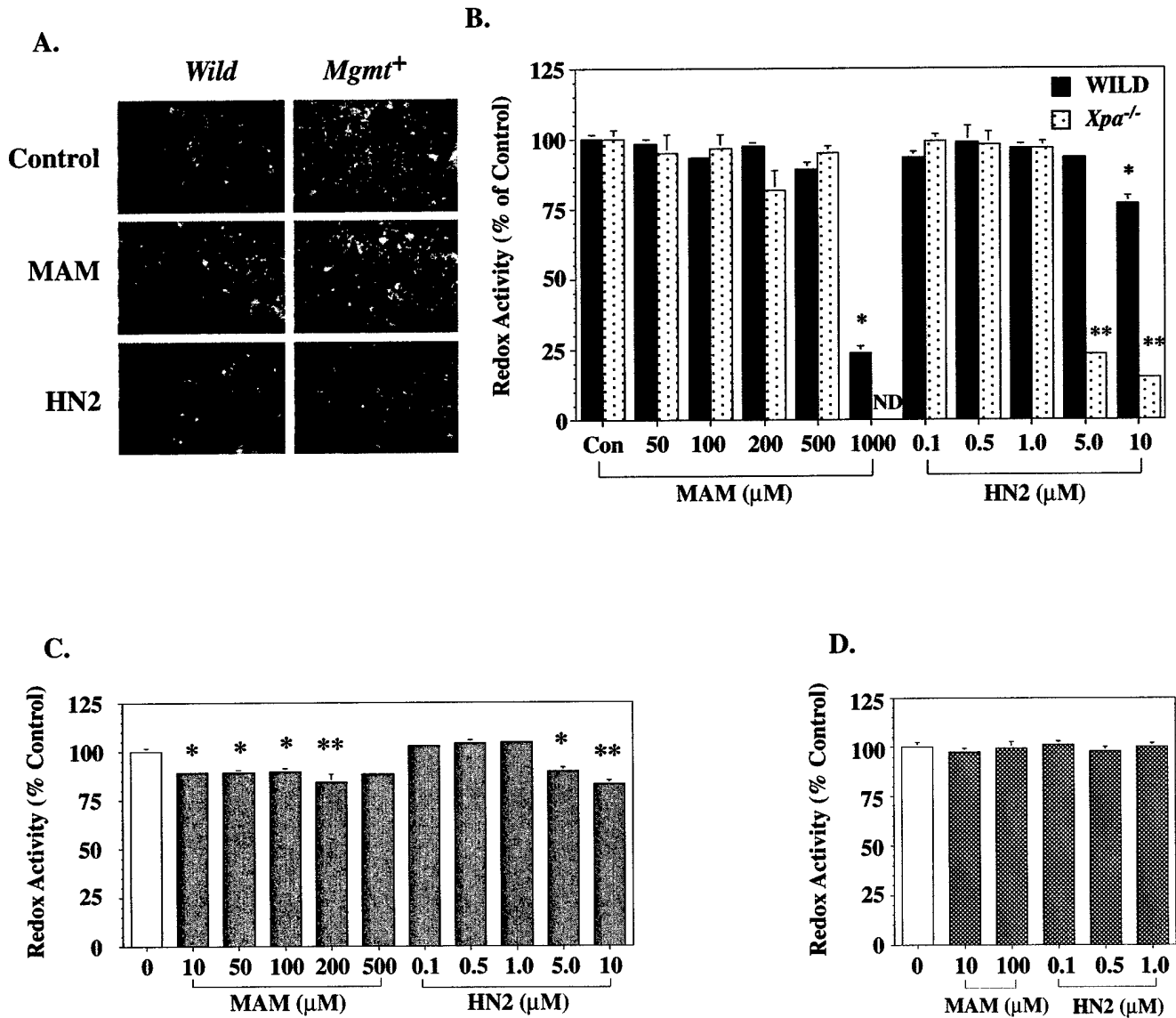
Figure 1. Viability of cerebellar neurons from wild type (Wild), *Mgmt*-overexpressing (*Mgmt*+) or *Xpa* /- mice [A,B] or cortical and midbrain neurons from wild type mice treated with HN2 or MAM [C,D]. Mouse cerebellar, cortical and midbrain neuronal cultures were treated with various concentrations of MAM (10 μM - 500 μM) or HN2 (0.1 μM - 10 μM) for 24h, the cultures incubated with Alamar Blue™ for 4h and examined for fluorescence. After 4h, the cultures were incubated with fluorochrome containing culture media (0.26 μM calcein-AM and 3.0 μM propidium iodide) and photomicrographs of representative fields from wild type and MGMT+ neuronal cell cultures treated with MAM or HN2 examined for viability by examining the cells for live (*green*) and dead (*red*) cells of fluorescent photomicrographs as previously described by Kisby *et al.* [24]. Values represent the mean ± SEM (*n*= *4*). Significantly different from toxin treated wild-type cells (* *p* < *0.05*, ** *p* <*0.01*, ANOVA). ND= not determined.

A.

B.

C.

D.
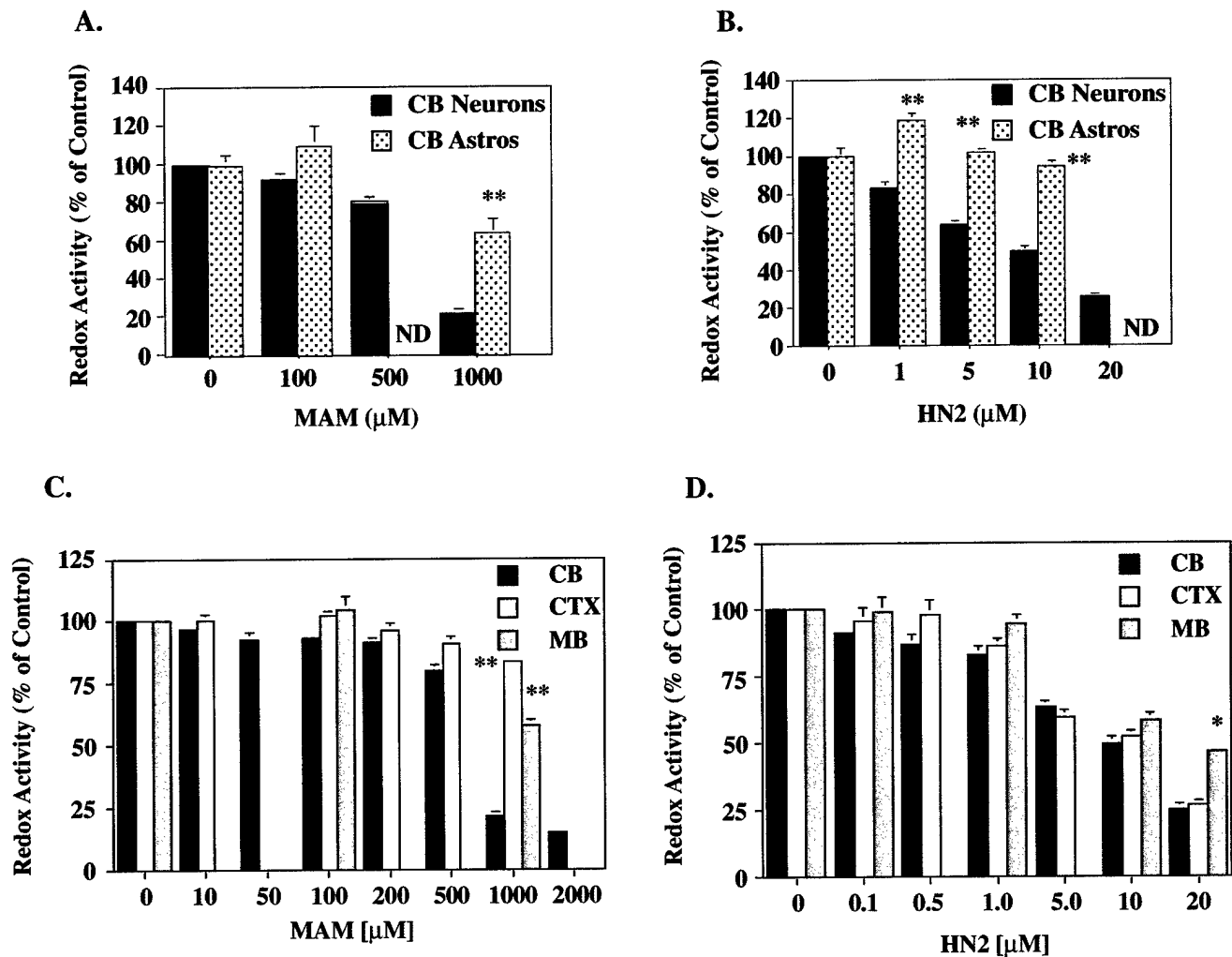
Figure 2. Comparative Toxicity of MAM and HN2 Among Different CNS Cell Types and Brain Regions. A,B. Primary neuronal and astrocyte cell cultures were prepared from the cerebellum of 6-7 day old C57BL/6 (wild type) mice and maintained for 7 or 14 days (respectively) before treatment for 24h with 100 μM-1000 μM methylazoxymethanol (MAM) or 1 μM-20 μM nitrogen mustard (HN2). **C,D.** Primary neuronal cell cultures were prepared from the cerebellum (CB), cerebral cortex (CTX) or midbrain (MB) of C57BL/6 (wild type) mice, the cultures maintained for 7d before treatment for 24h with 10 μM-2000 μM MAM or 0.1 μM-20 μM HN2. After toxin treatment, all cultures were incubated with Alamar Blue™ for 4h and examined for fluorescence. Values represent the mean % redox activity of controls ± SEM of 3-6 separate experiments (*n= 4*). Significantly different from CB neurons (** *p* <*0.001*, ANOVA) or CB and CTX neurons (* *p* < *0.05*, ANOVA). ND= not determined.
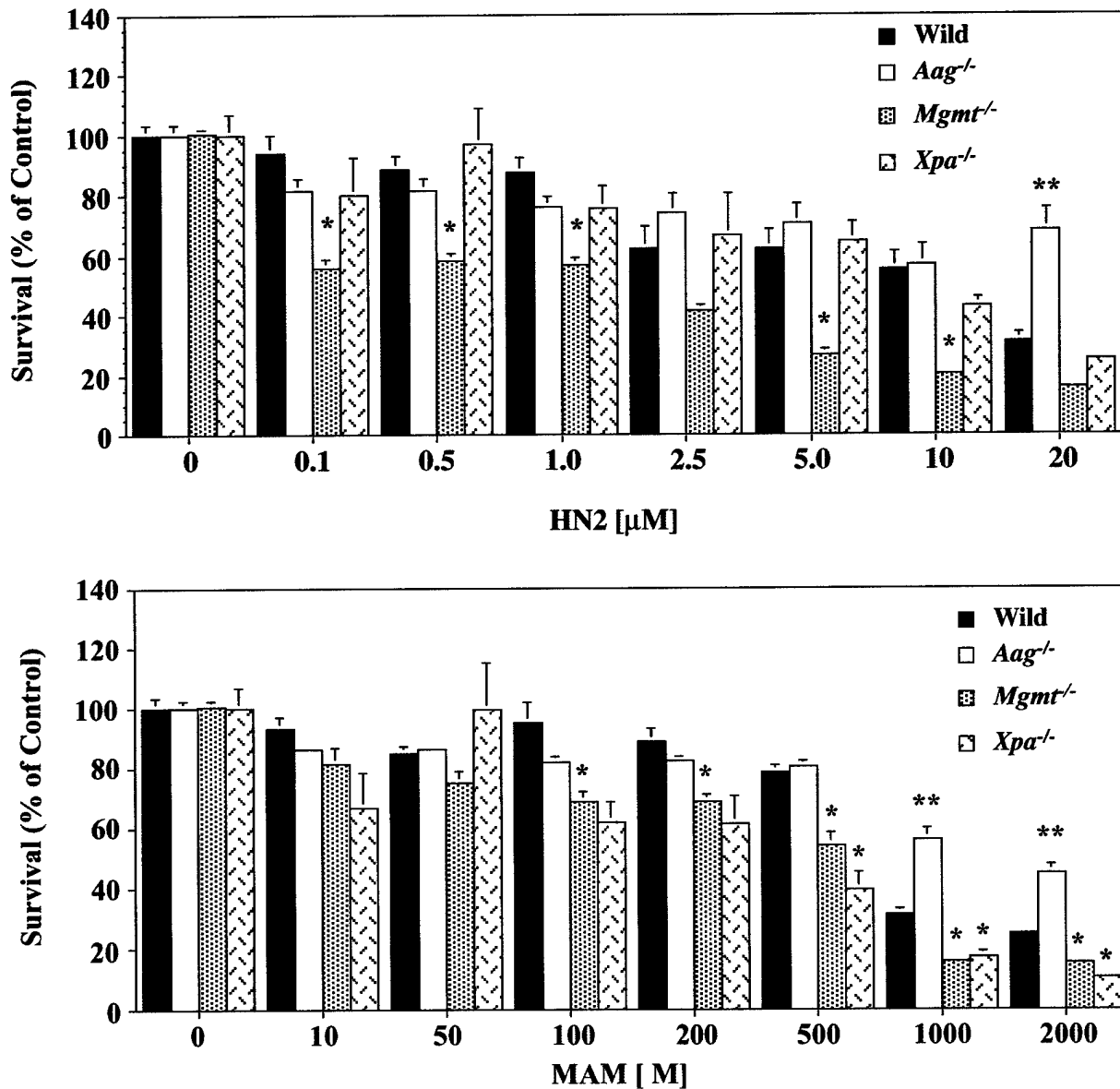
Figure 3. Viability of DNA Repair-Deficient Cerebellar Neurons Treated with HN2 or MAM.
Mouse cerebellar granule cell cultures were treated with various concentrations of MAM (10 μM-2000 μM) or HN2 (0.1 μM-20 μM) for 24h, incubated with calcein-AM and examined for fluorescence. Values represent the mean % survival of controls ± SEM. Significantly different from toxin treated wild-type cells (* $p < 0.01$, ** $p < 0.001$, ANOVA).
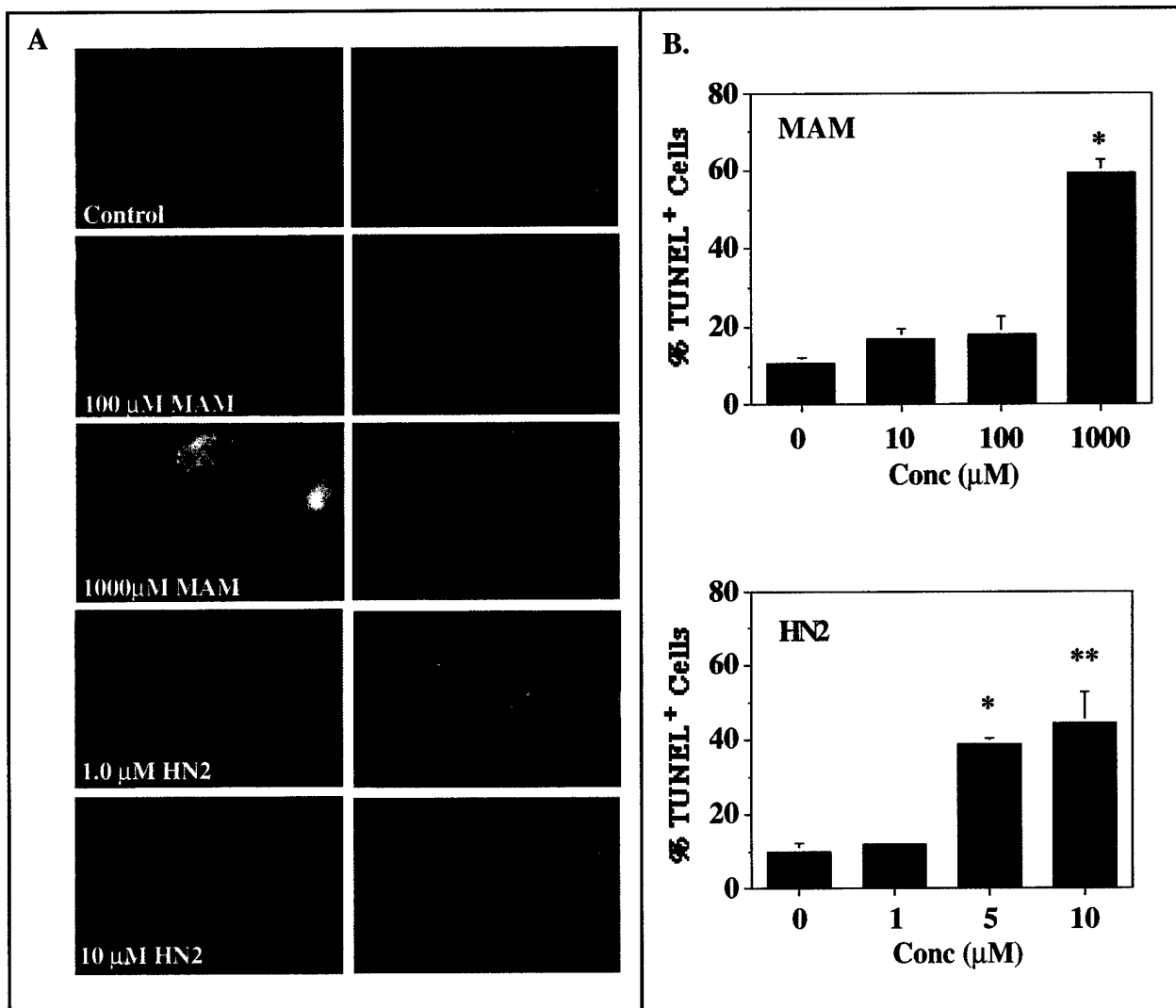
Figure 4. Markers of Apoptosis in MAM and HN2 Treated Wild Type (C57 BL/6) Neurons.
Representative fluorescent micrographs from primary cerebellar neurons treated with various concentrations of MAM (100 μM and 1000 μM) or HN2 (1.0 μM and 10 μM) for 24h, and the cultures examined for the integrity of the mitochondrial membrane potential using the fluorescent dye DePsipher™ (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethybenzimidazolyl carbocyanine iodide or JC-1, Trevigen Inc.) and the nuclear membrane (Hoeschst 33342) [A] or the cells examined for DNA damage using the NeuroTacs™ kit (Trevigen, Inc.) [B]. The NeuroTacs™ kit detects DNA strand breaks in cells by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique. Fluorescent orange-red aggregates accumulate in mitochondria with an intact membrane potential while the green monomer accumulates in cells with a perturbed mitochondrial membrane potential. Note the marked effect of HN2 (10 μM) on the mitochondrial membrane potential when compared with MAM treated neurons (compare *top* and *bottom* panels). Note also the lack of extensive nuclear staining for Hoechst (bright blue fluorescence) for MAM or HN2 treated neurons. Significantly different from controls (* $p < 0.01$, ** $p < 0.001$, ANOVA).
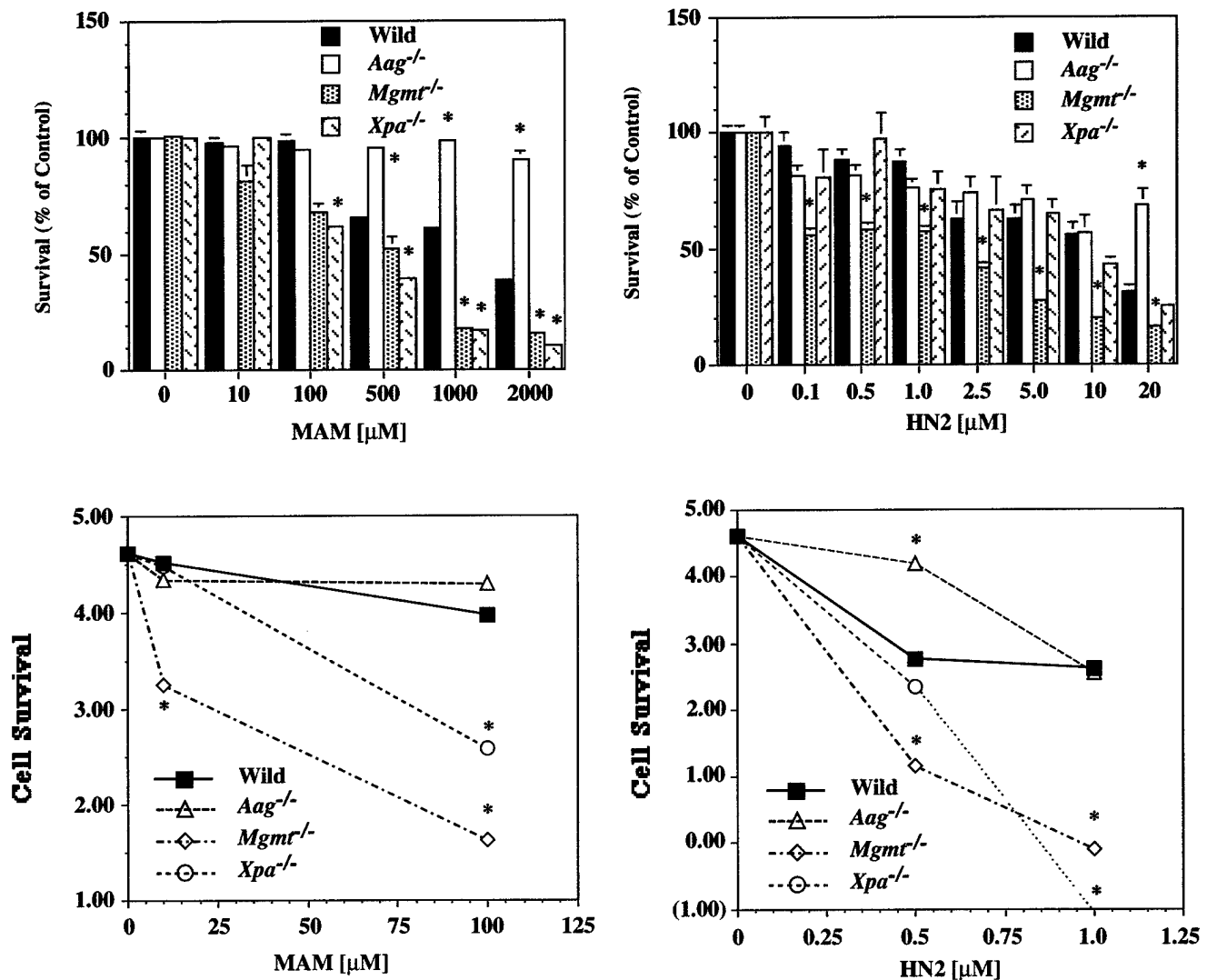
Figure 5. Viability of Wild-Type (C57BL6), $Mgmt^{-/-}$, $Aag^{-/-}$, **and** $Xpa^{-/-}$ **mouse skin fibroblasts treated with MAM.** Skin fibroblast cell lines were developed from the ears of wild type, $Mgmt^{-/-}$, $Aag^{-/-}$, and $Xpa^{-/-}$ mice, the cells seeded at a density of 30,000 cells/well (short-term studies, *top*) or 4000 cells/dish (long-term studies, *bottom*). The cultures were treated for 24h with various concentrations of HN2 (1.0 μM - 20 μM) or MAM (100 μM - 1000 μM) and the cells either incubated with Alamar Blue™ for 4h and examined for viability (*short-term studies*) or the toxin media removed, replaced with control culture media and the cloning efficiency of 2-3 week old cultures determined on crystal violet stained culture dishes (*long-term studies*). Values represent the mean ± SEM (*n*= 2 experiments). Significantly different from wild-type cells (*p <0.001*, ANOVA).
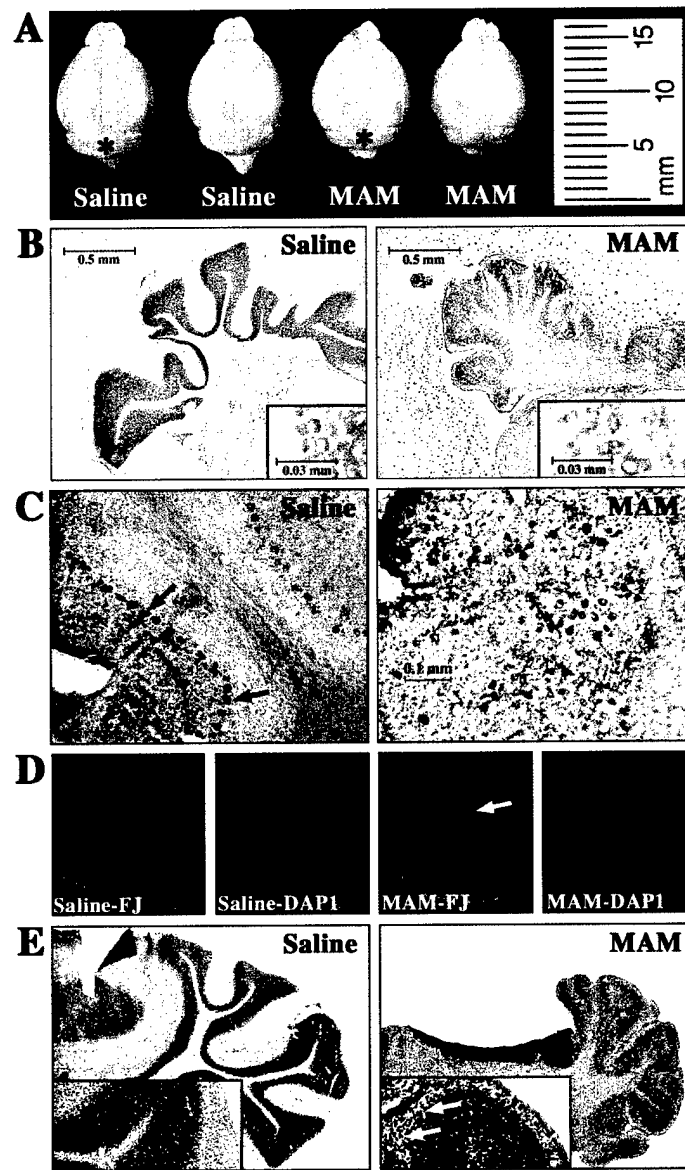
Figure 6. Morphology of the Cerebellum from Postnatal C57BL/6 Mice treated with MAM. Gross observation of the brain of MAM treated mice and light micrographs of representative areas from the cerebellum of 21 day-old pups treated at postnatal day 1-2 with saline (control) or MAM (30 mg/kg, s.c.) [A]. Note the smaller cerebellum (*stars*) of MAM *vs.* saline treated mice (A), but similar size of the respective cerebral hemispheres. [B-E] Fluorescent and light micrographs of representative areas from coronal sections (25 μm) of the cerebellum of 21 day-old pups treated at postnatal day 1-2 with saline (SAL) or MAM (30 mg/kg, s.c.). At the light microscopic level (B), cresyl violet stained sections revealed smaller cerebellar folia (F), thinner cerebellar cortex and disorganization of neurons in the granule (GL), Purkinje (PL) and molecular (ML) layers (*inset* in B). CaBP was particularly useful for visualizing the disorganization (*arrows*) and abnormal appearance of Purkinje cells in MAM treated animals (C). Neuronal degeneration was determined by examining tissue sections incubated with the anionic fluorescein dye Fluoro-Jade B™ (FJ, Histo-Chem Inc) and the nuclear stain DAPI [D] or silver stain (NeuroSilver™, FD Technologies) [E] according to the manufacturer's protocols. Note the intense fluorescence of some neurons in the granule cell layer (*white arrow* in D*)* and the intense precipitate over silver stained cerebellar neurons (*white arrows,* in E) in MAM treated animals.

A.
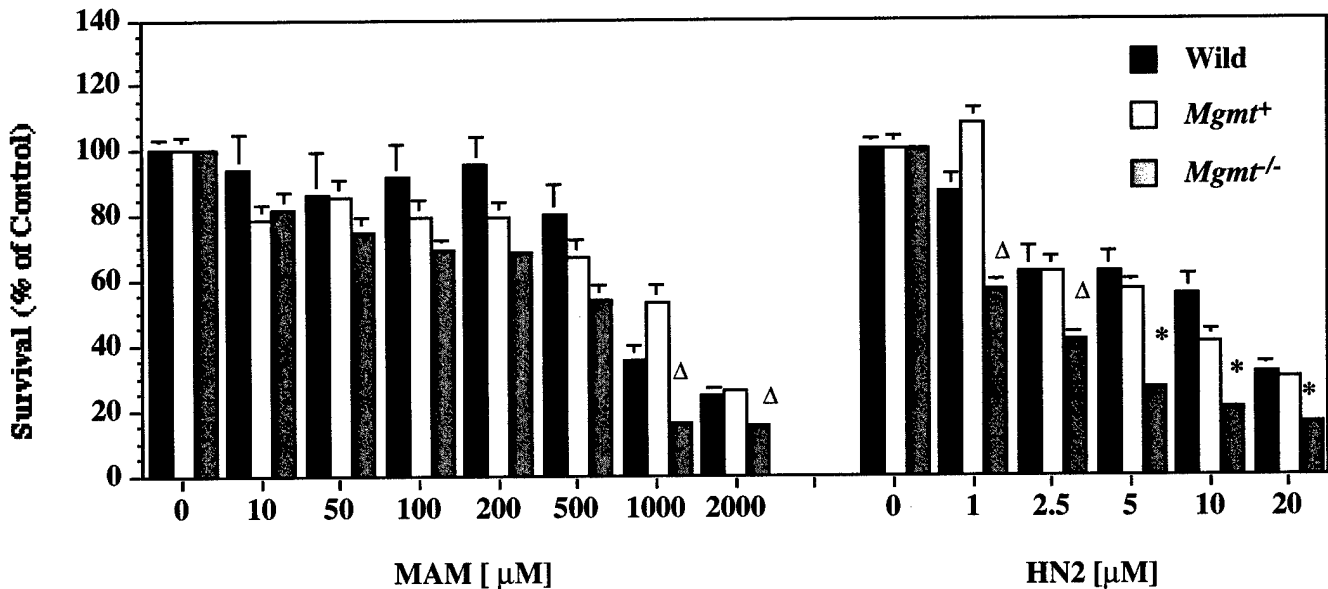
B.
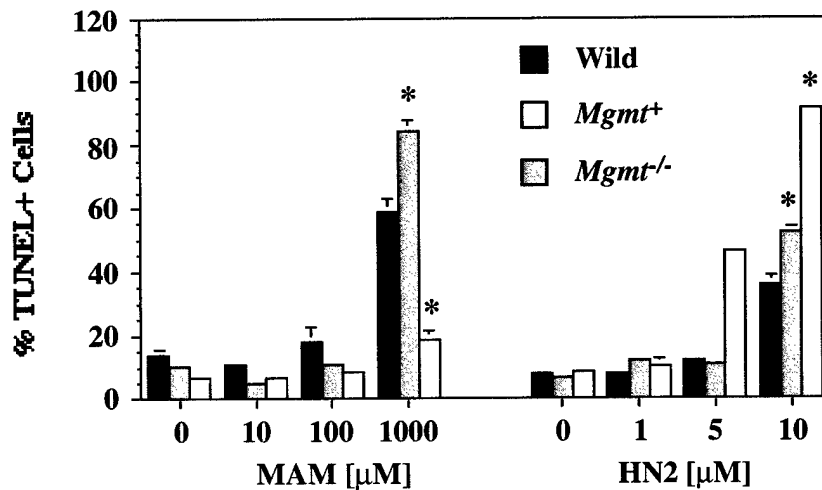
Figure 7. Influence of MGMT on MAM and HN2-Induced Neurotoxicity. Mouse cerebellar granule cell cultures were treated with various concentrations of MAM (10 μM-2000 μM) or HN2 (1.0 μM-20 μM) for 24h, incubated with calcein-AM and examined for fluorescence. Values represent the mean % survival of controls ± SEM. Significantly different from toxin treated MGMT$^{-/-}$ cells (* p <0.05, † p < 0.01, Δ p <0.001, ANOVA).
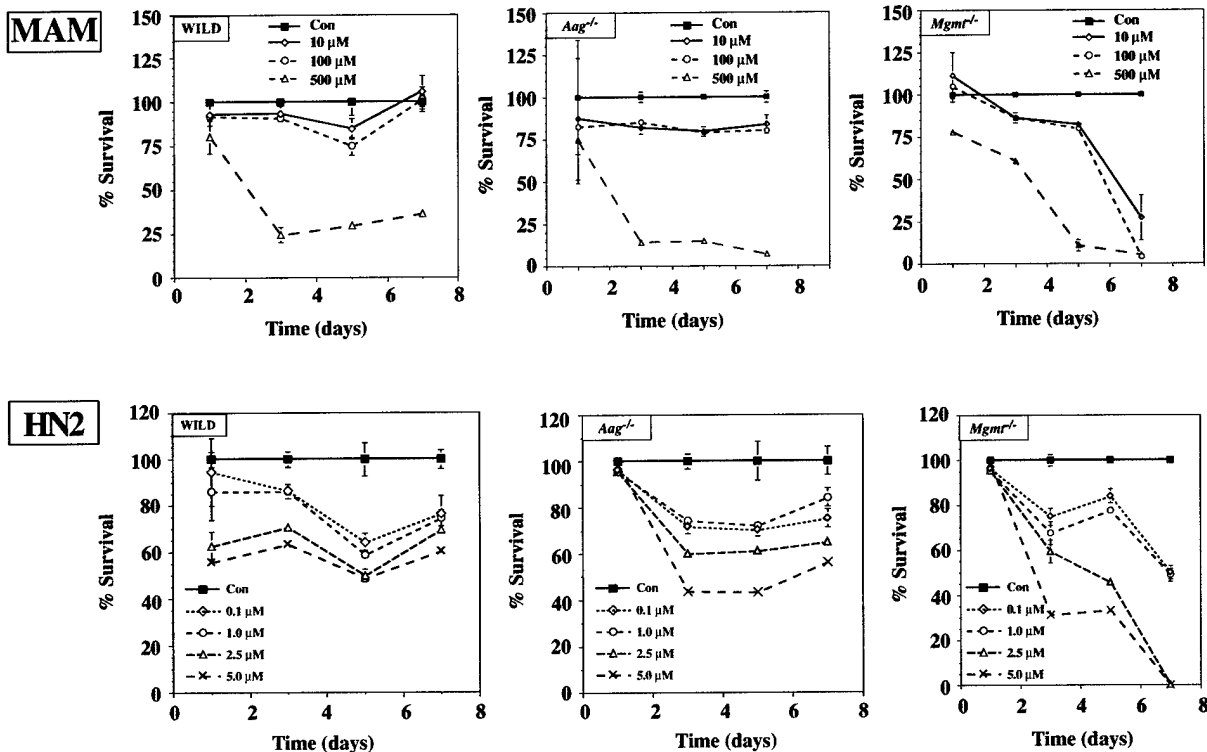
Figure 8. Long Term Viability of Neurons Treated with MAM or HN2. Mouse cerebellar granule cell cultures were treated continuously with HN2 (0.1 μM-5.0 μM) or MAM (10 μM-500 μM) and, at various time periods (1, 3, 5, 7 days), the cell cultures incubated with calcein-AM and examined for fluorescence. Values represent the mean % survival of controls ± SEM (n= 6/group, 2-3 separate experiments).
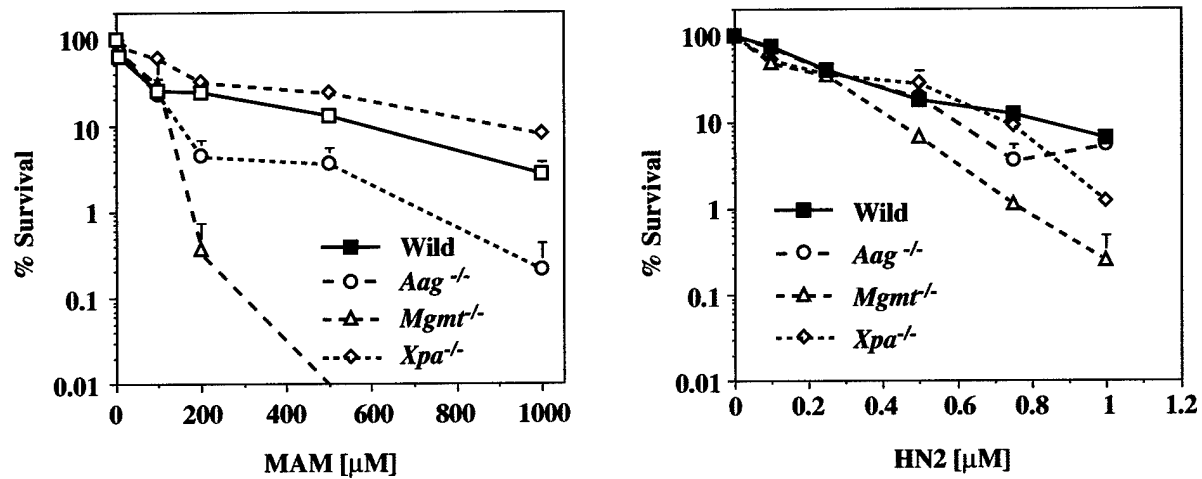
Figure 9. Cloning Efficiency of DNA Repair-Deficient Kidney Cells Treated with MAM or HN2.
Mouse kidney epithelial cell cultures were treated for 24h with MAM (10 μM and 1000 μM) or HN2 (0.1 μM-1.0 μM), the cell culture media replaced with toxin-free media and after 2-3 weeks *in vitro* (75-80% confluency), the cell cultures stained with cresyl violet and the number of colonies manually counted. Values represent the mean ± SEM from three separate experiments. Significantly different from HN2 or MAM treated wild-type cells.
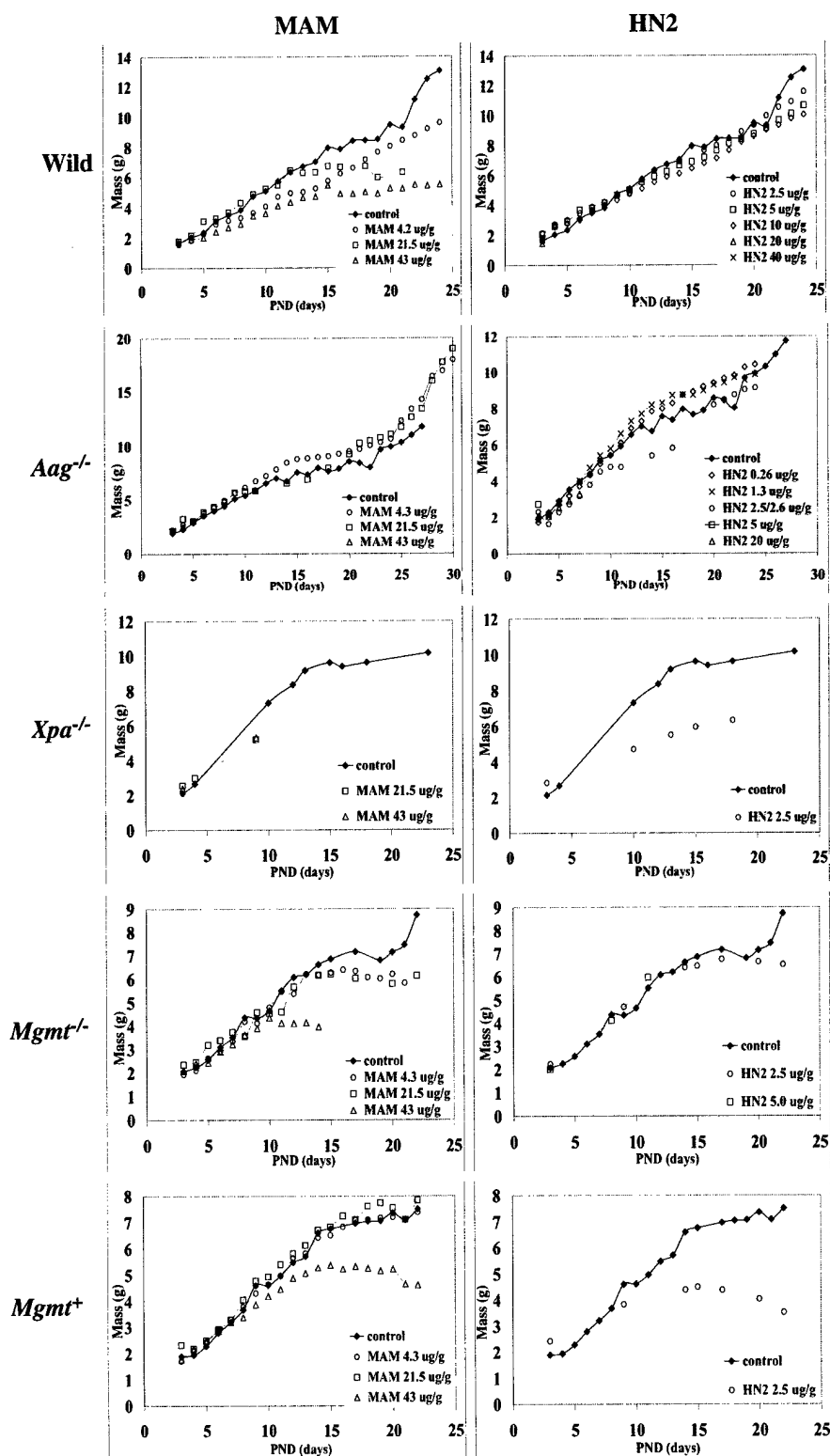
Figure 10. Growth of wild type and DNA repair mutant mice after a single injection of saline, MAM (4.3 mg/kg to 43 mg/kg, s.c.) or HN2 (0.26 mg/kg to 40 mg/kg).
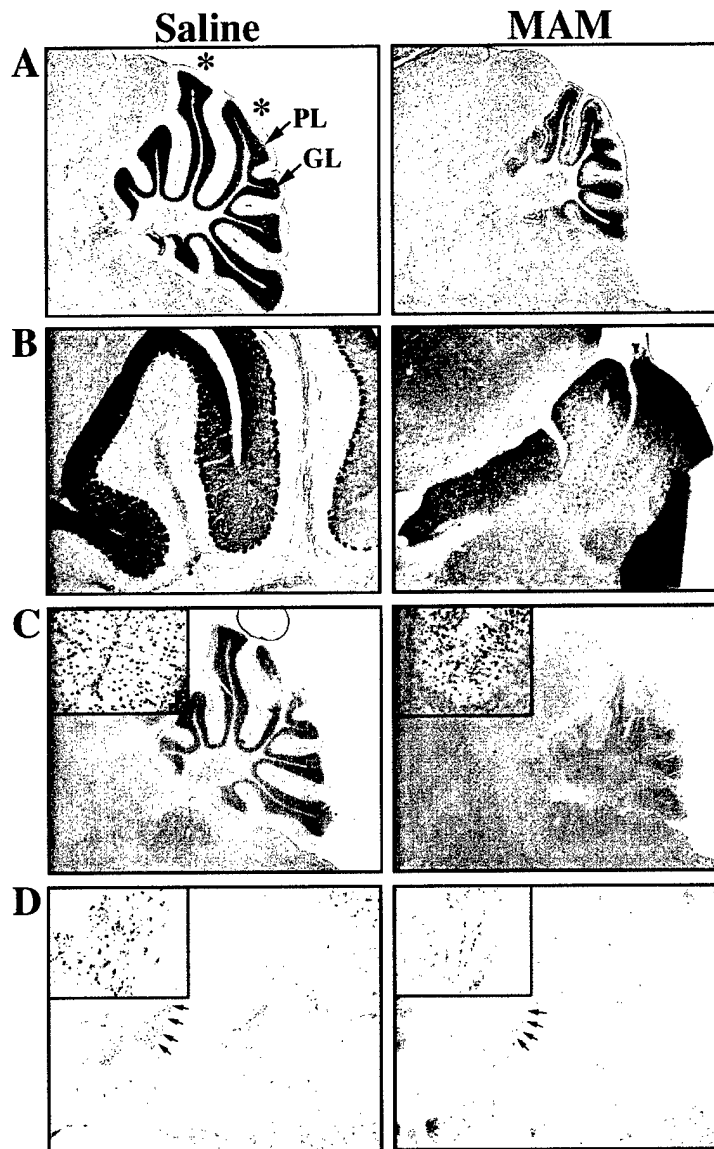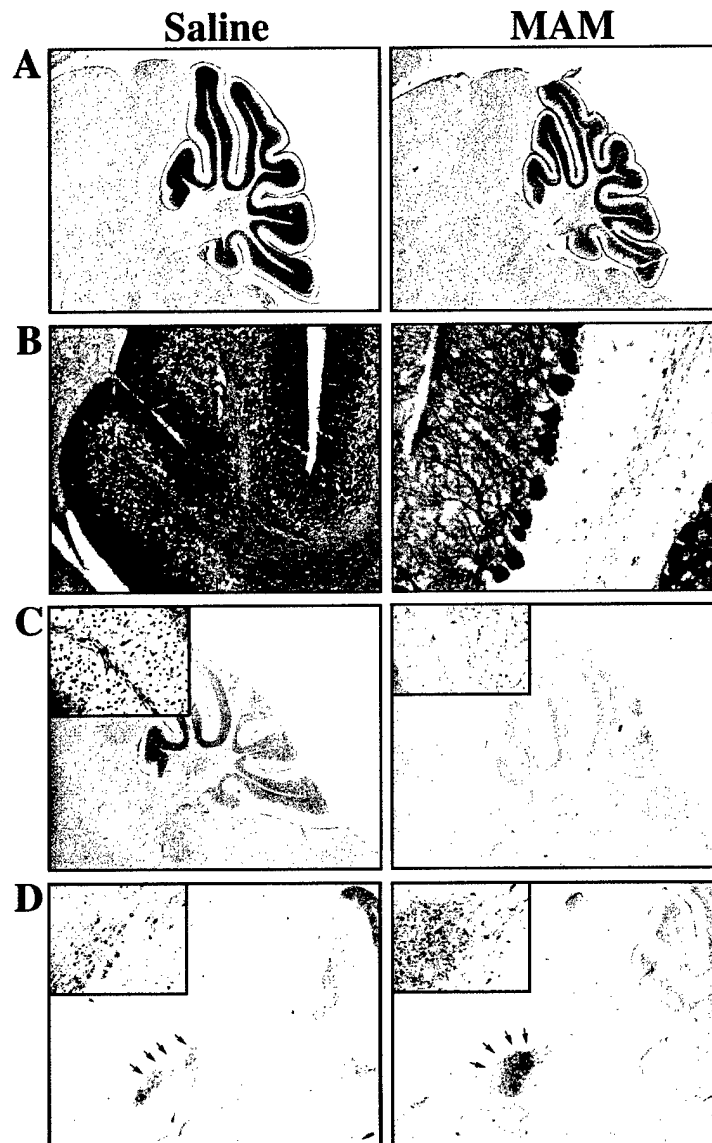
Figure 11. Neuropathology of the Brain from Postnatal C57BL/6 Mice treated with MAM. Light micrographs of representative areas from sagittal sections (25 µm) of the cerebellum [A,B,C] or midbrain [D] of 22 day-old pups treated at postnatal day 3 with saline or MAM (21.5 mg/kg, s.c.). Neuronal degeneration was determined by examining tissue sections incubated with silver stain (NeuroSilver™, FD Technologies) according to the manufacturer's protocols. Alternate tissue sections were immunoprobed with antibodies to the calcium-binding protein calbindin (**B**) or to tyrosine hydroxylase to label dopaminergic cells (**D**). Note the smaller cerebellum of MAM vs. saline treated mice (**A**) in cresyl violet stained sections. At the light microscopic level (**B**), smaller cerebellar folia (F), thinner cerebellar cortex and disorganization of neurons in the granule (GL), Purkinje (PL) and molecular (ML) layers were observed. CaBP was particularly useful for visualizing the disorganization and abnormal appearance of Purkinje cells within the cerebellum of MAM treated animals (**B**). Note also the reduced staining for tyrosine hydroxylase in nigral neurons (**D**) and the preservation of the neuronal organization (vs. Mgmt$^{-/-}$ neurons, *compare with* **Figure 13D**) within the midbrain of MAM treated mice.
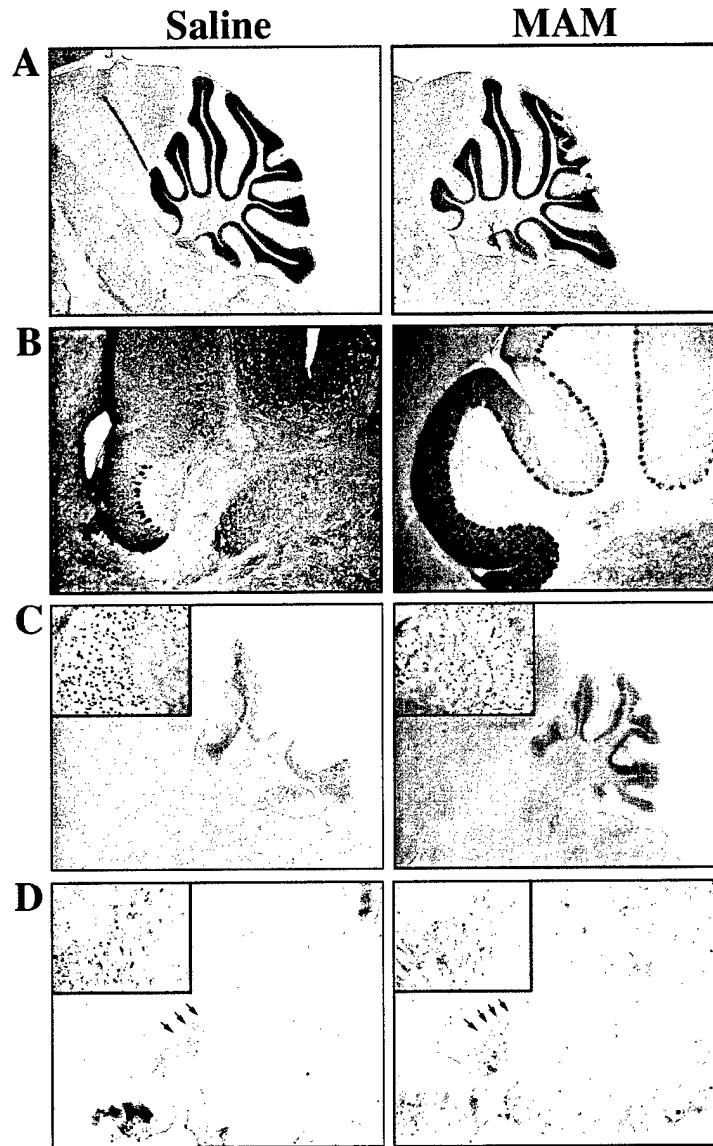
Figure 12. Neuropathology of the Brain from Postnatal *Aag*^{-/-} **Mice treated with MAM.** Light micrographs of representative areas from sagittal sections (25 µm) of the cerebellum [A,B,C] or midbrain [D] of 22 day-old pups treated at postnatal day 3 with saline or MAM (21.5 mg/kg, s.c.). Neuronal degeneration was determined by examining tissue sections incubated with silver stain (NeuroSilver™, FD Technologies) according to the manufacturer's protocols. Alternate tissue sections were immunoprobed with antibodies to the calcium-binding protein calbindin (**B**) or to tyrosine hydroxylase to label dopaminergic cells (**D**). Note the preservation of the cerebellum of MAM *vs.* saline treated mice (**A**) in cresyl violet stained sections when compared to moderately dosed *Mgmt*^{-/-} mice (*see* **Figure 13A**). At the light microscopic level (**B**), the cerebellar folia and the organization of neurons in the granule, Purkinje and molecular layers were preserved in CaBP (**B**) and silver stained (**C**) tissue sections of MAM treated mice. Note also the organization and staining intensity of tyrosine hydroxylase immunoreactive neurons nigral neurons (**D**) is preserved within the midbrain of MAM treated mice.
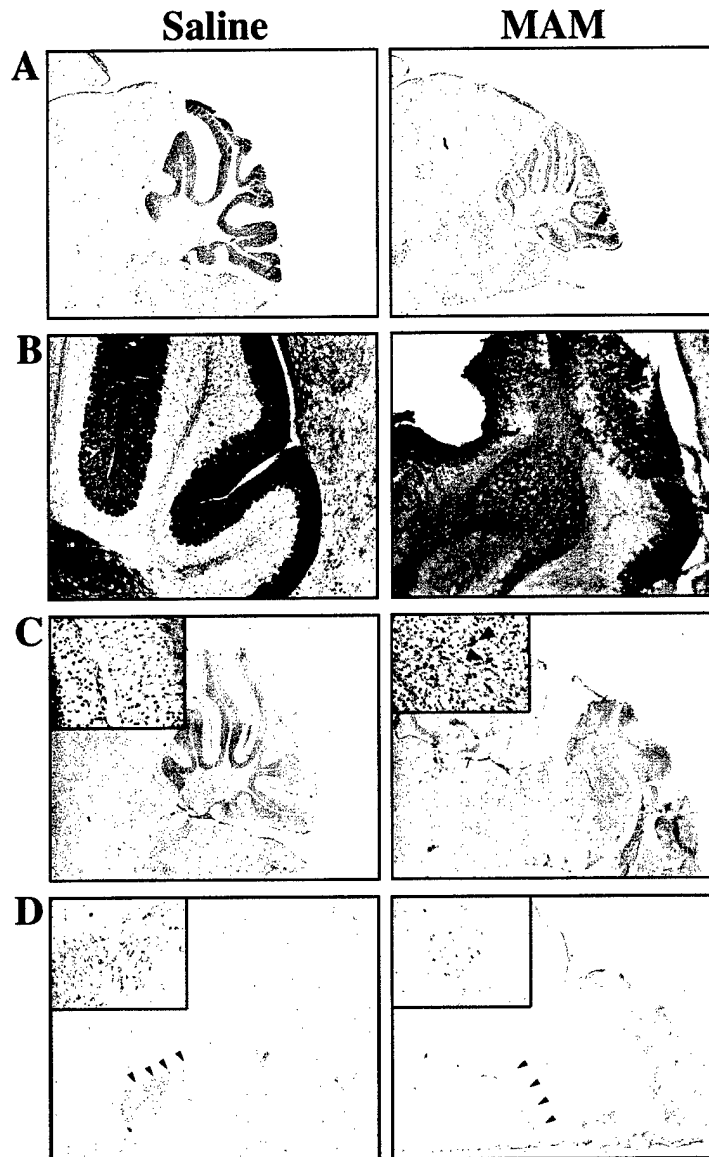
Figure 13. Neuropathology of the Brain from Postnatal *Mgmt*^{-/-} Mice treated with MAM. Light micrographs of representative areas from sagittal sections (25 μm) of the cerebellum [**A,B,C**] or midbrain [**D**] of 22 day-old pups treated at postnatal day 3 with saline or MAM (21.5 mg/kg, s.c.). Neuronal degeneration was determined by examining tissue sections incubated with silver stain (NeuroSilver™, FD Technologies) according to the manufacturer's protocols. Alternate tissue sections were immunoprobed with antibodies to the calcium-binding protein calbindin (**B**) or to tyrosine hydroxylase to label dopaminergic cells (**D**). Note the smaller cerebellum of MAM *vs.* saline treated mice (**A**) in cresyl violet stained sections. At the light microscopic level (**B & C**), smaller cerebellar folia, thinner cerebellar cortex and disorganization and degeneration of neurons in the granule, Purkinje and molecular layers were observed. CaBP was particularly useful for visualizing the disorganization and abnormal appearance of Purkinje cells within the cerebellum of MAM treated animals (**B**). Note also the reduced staining for tyrosine hydroxylase and the abnormal organization of nigral neurons (**D**) within the midbrain of MAM treated mice.
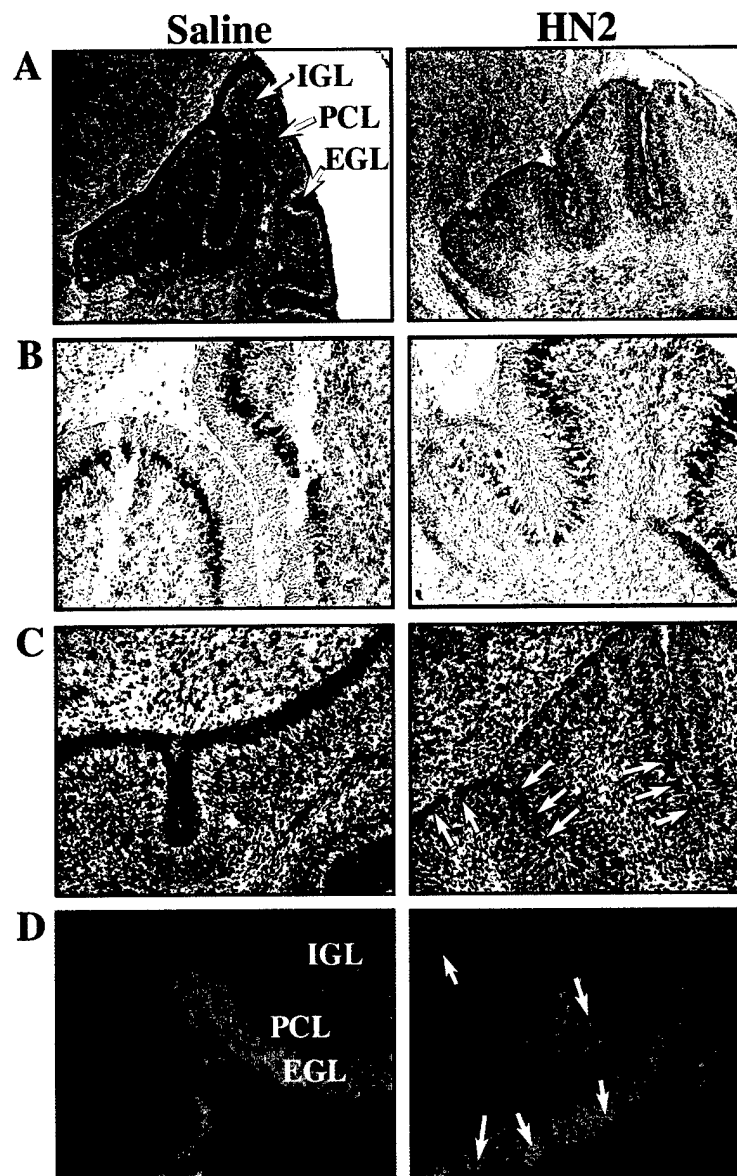
Figure 14. Neuropathology of the Brain from Postnatal *Mgmt+* Mice treated with MAM. Light micrographs of representative areas from sagittal sections (25 μm) of the cerebellum [A,B,C] or midbrain [D] of 22 day-old pups treated at postnatal day 3 with saline or MAM (43 mg/kg, s.c.). Neuronal degeneration was determined by examining tissue sections incubated with silver stain (NeuroSilver™, FD Technologies) according to the manufacturer's protocols. Alternate tissue sections were immunoprobed with antibodies to the calcium-binding protein calbindin (B) or to tyrosine hydroxylase to label dopaminergic cells (D). Note the mild atrophy of the cerebellum of MAM *vs.* saline treated mice (A) in cresyl violet stained sections when compared to moderately dosed *Mgmt-/-* mice (*see* **Figure 13A**). At the light microscopic level (B), the cerebellar folia and the organization of neurons in the granule, Purkinje and molecular layers were preserved in CaBP (B) and silver stained (C) tissue sections of MAM treated mice. Note also the organization and staining intensity of tyrosine hydroxylase immunoreactive neurons nigral neurons (D) is preserved within the midbrain of MAM treated mice.
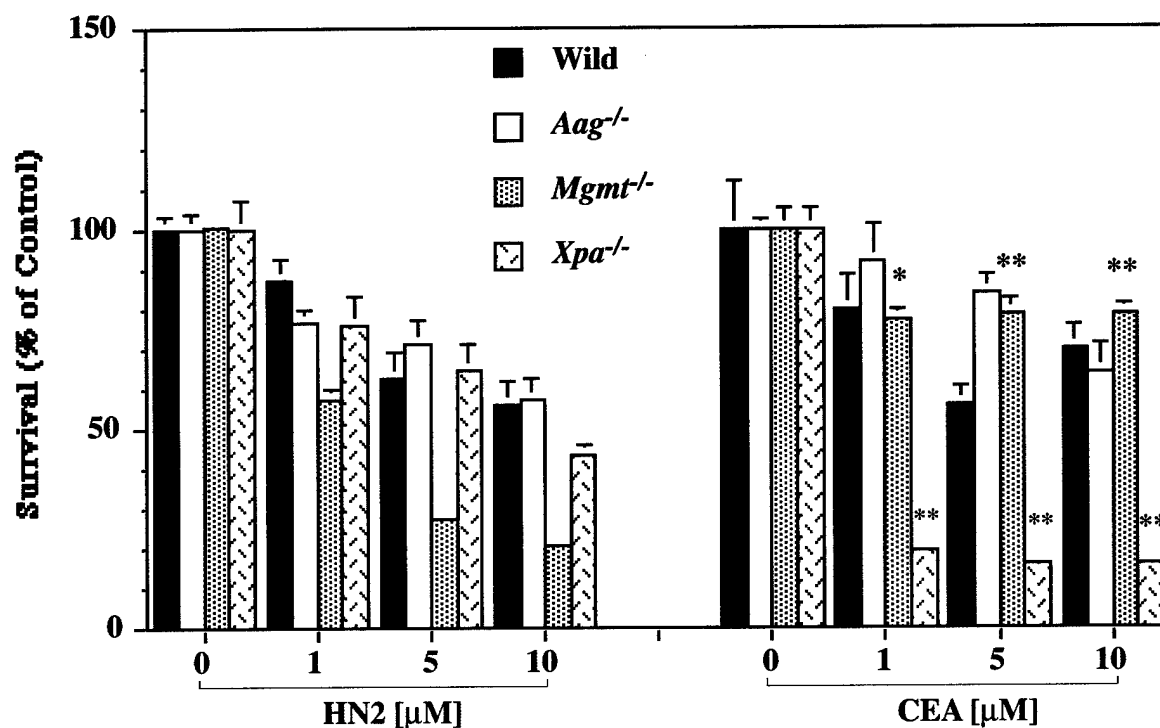
Figure 15. Viability of DNA Repair Deficient Cerebellar Neurons Treated with mechlorethamine (HN2) or the monofunctional nitrogen mustard 2-chloroethylamine (CEA). Cerebellar granule cell cultures from C57BL/6J mice were treated with various concentrations (0.1 μM-20 μM) of HN2 or CEA for 24h, incubated with calcein-AM and examined for fluorescence. Values represent the mean % survival of controls ± SEM (*n=6*, 2-3 separate experiments). Significantly different from HN2 treated cells (* *p* < *0.01*, ** *p* <*0.001*, ANOVA).

A.

B.
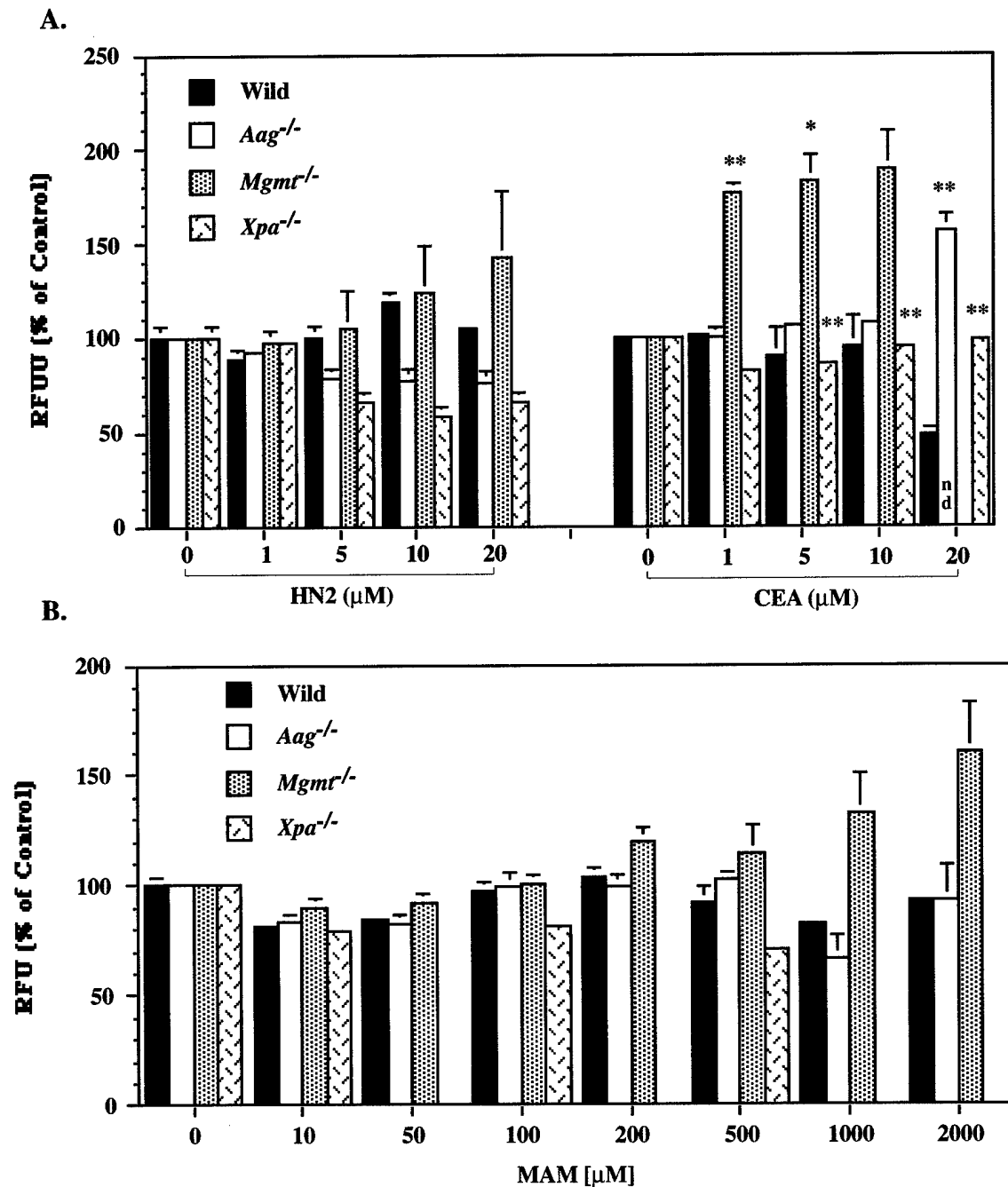
Figure 16. Intracellular Glutathione (GSH) Levels in Wild Type and DNA Repair-Deficient Neurons Treated with HN2 or CEA. Mouse cerebellar granule cell cultures were treated with various concentrations (1.0 μM to 20 μM) of HN2 or CEA for 24h, the cultures incubated with the GSH specific fluorophore monochlorobimane hydrochloride (40 μM) [5,40], and the cells examined on a fluorescence microplate reader. Values represent the mean relative fluorescence intensity units (RFU) ± SEM ($n=6$, 2 separate experiments). Significantly different from toxin treated wild-type cells (*$p < 0.01$, **$p < 0.001$). **nd** = not determined.
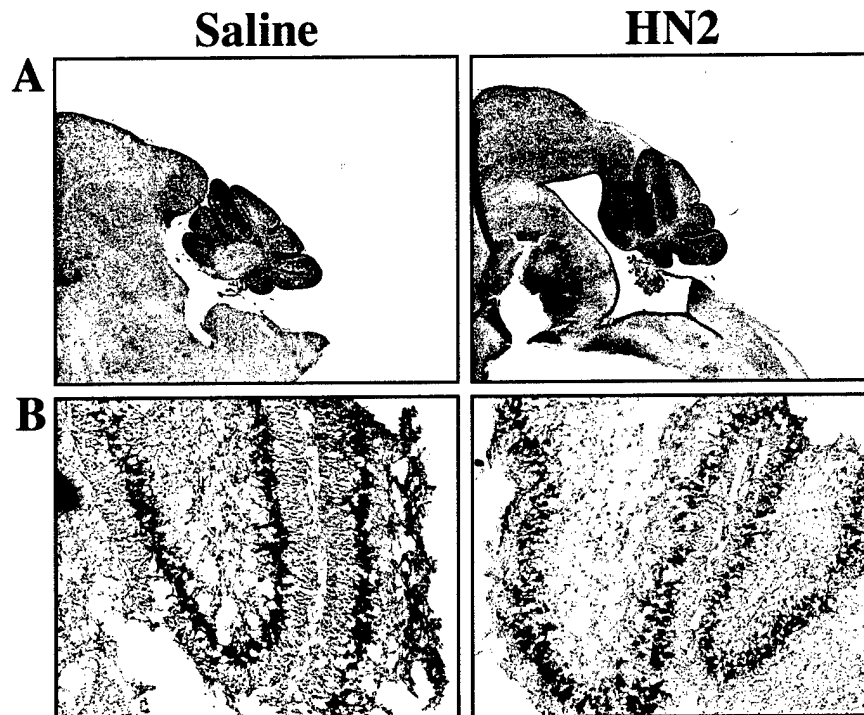
Figure 17. Neuropathology of the Cerebellum from Young C57BL/6 Mice treated with HN2.
Light micrographs of representative areas from sagittal sections (25 μm) of the cerebellum [A-D] of 4 day-old pups treated at postnatal day 3 with saline or HN2 (5 mg/kg, s.c.). The cytoarchitecture of the cerebellum was determined by staining tissue sections with cresyl violet [A] or immunoprobing with an antibody to calbindin [B] while the extent of degenerating neurons was determined by staining alternate sections with either silver stain (NeuroSilver™, FD Technologies) [C] or FluoroJade B/Hoescht 33342 [D]. Note the smaller cerebellum of HN2 vs. Saline treated mice in cresyl violet stained sections [A] and the presence of heavy silver deposits (*arrows in* C) or increased yellow fluorescence (*arrows in* D) over degenerating neurons within the external (EGL) and internal (IGL) granule cell layer. At the light microscopic level [A], smaller cerebellar folia, thinner cerebellar cortex and disorganization of neurons in the external granule (EGL), Purkinje (PCL) and internal granule (IGL) cell layers were also observed.
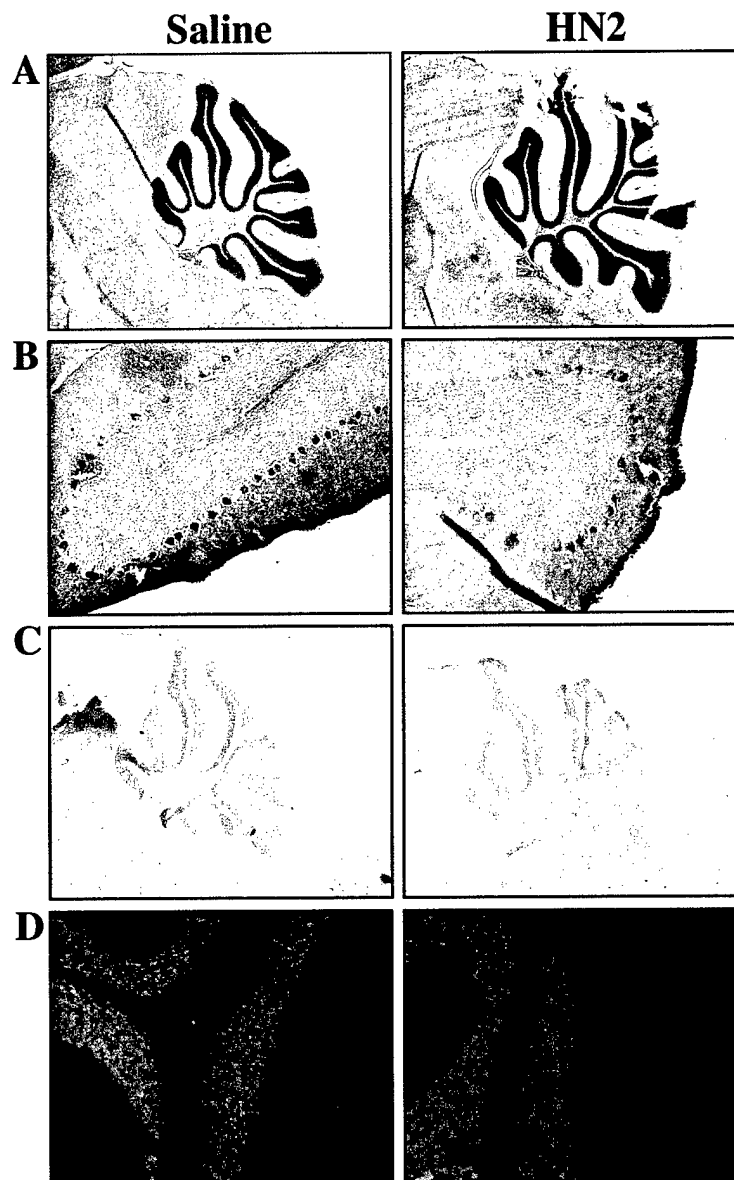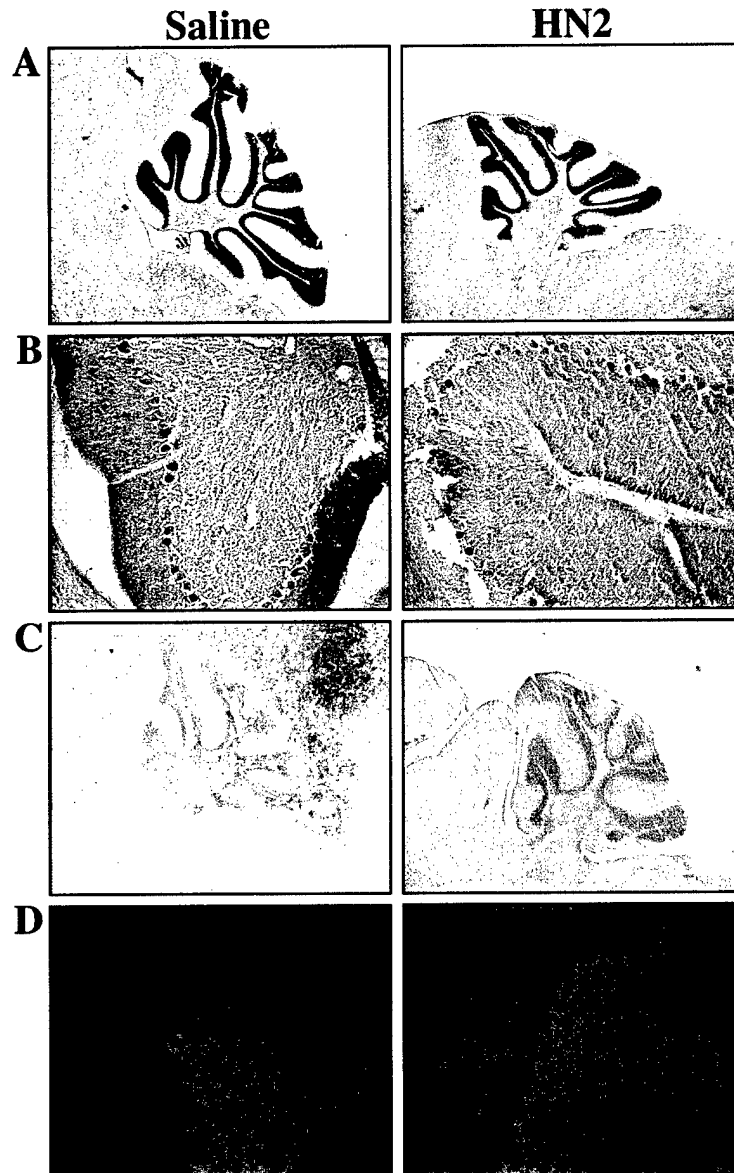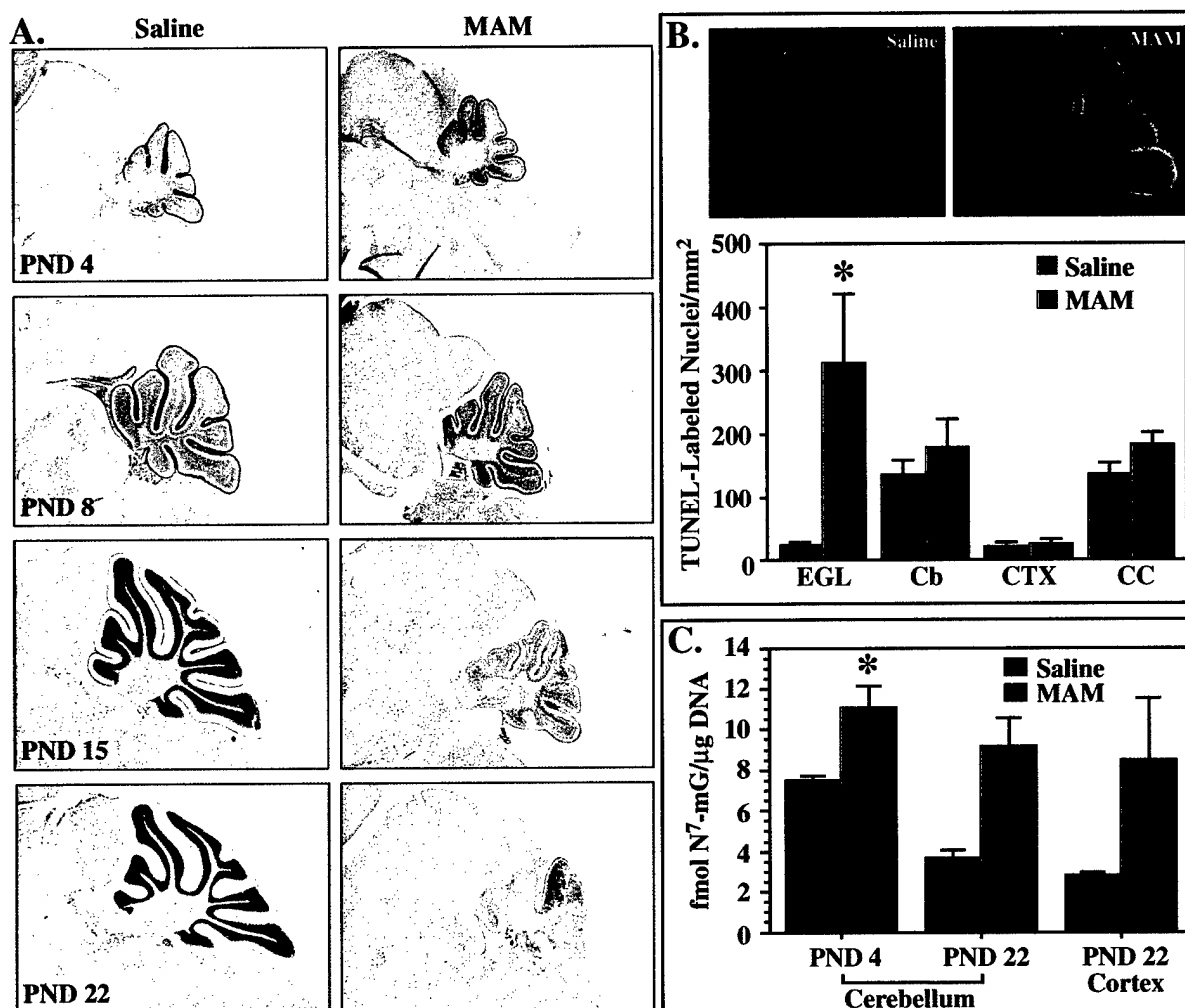
Figure 18. Neuropathology of the Cerebellum from Young Aag$^{-/-}$ Mice treated with HN2. Light micrographs of representative areas from sagittal sections (25 μm) of the cerebellum [A-D] of 22 day-old pups treated at postnatal day 3 with saline or HN2 (5 mg/kg, s.c.). The cytoarchitecture of the cerebellum was determined by staining tissue sections with cresyl violet [A] or immunoprobing with an antibody to calbindin [B] while the extent of degenerating neurons was determined by staining alternate sections with either silver stain (NeuroSilver™, FD Technologies) [C] or FluoroJade B/Hoescht 33342 [D]. Note the preservation of the cerebellum of HN2 vs. Saline treated mice in cresyl violet stained sections [A] and the lack of silver deposits [C] or increased yellow fluorescence [D] over degenerating neurons within the external (EGL) and internal (IGL) granule cell layer. At the light microscopic level, the cerebellar folia and the organization of neurons in the granule, Purkinje and molecular layers were preserved in cresyl violet stained [A] and CaBP [B] immunoprobed tissue sections of HN2 treated mice.

Figure 19. Neuropathology of the Cerebellum from Young *Mgmt*^{-/-} Mice treated with HN2. Light micrographs of representative areas from sagittal sections (25 μm) of the cerebellum [**A,B**] of 4 day-old pups treated at postnatal day 3 with saline or HN2 (5 mg/kg, s.c.). The cytoarchitecture of the cerebellum was determined by staining tissue sections with cresyl violet [**A**] or immunoprobing with an antibody to calbindin [**B**]. Note that the cerebellum of HN2 *vs*. Saline treated mice in cresyl violet stained sections is similar 24h after dosing with HN2 [**A**]. At the light microscopic level, the cerebellar folia and the organization of neurons in the granule, Purkinje and molecular layers were preserved in cresyl violet stained [**A**] and CaBP [**B**] immunoprobed tissue sections of HN2 treated mice.

Figure 20. Neuropathology of the Cerebellum from Young $Xpa^{-/-}$ **Mice treated with HN2.** Light micrographs of representative areas from sagittal sections (25 μm) of the cerebellum [A-H] of 22 day-old pups treated at postnatal day 3 with saline or HN2 (5 mg/kg, s.c.). The cytoarchitecture of the cerebellum was determined by staining tissue sections with cresyl violet [A] or immunoprobing with an antibody to calbindin [B] while the extent of degenerating neurons was determined by staining alternate sections with either silver stain (NeuroSilver™, FD Technologies) [C] or FluoroJade B/Hoescht 33342 [D]. Note the preservation of the cerebellum of HN2 vs. Saline treated mice in cresyl violet stained sections [A] and the lack of silver deposits [C] or increased yellow fluorescence [D] over degenerating neurons within the external (EGL) and internal (IGL) granule cell layer. Note the similarities with Aag$^{-/-}$ mice treated with the same concentration of HN2 (*compare with* **Fig. 18A,C,D**). At the light microscopic level, the cerebellar folia and the organization of neurons in the granule, Purkinje and molecular layers were preserved in cresyl violet stained [A] and CaBP [B] immunoprobed tissue sections of HN2 treated mice.

Figure 21. The cytoarchitecture and extent of DNA damage in the brain of C57BL/6 mice treated with MAM. A). Light micrographs of representative areas from cresyl violet stained parasagittal sections (20 μm) of the cerebellum from 4 day-, 8 day-, 15 day- and 22 day-old pups treated at postnatal day 3 (PND3) with saline (*left panels*) or MAM (43 mg/kg, s.c.)(*right panels*). B). DNA fragmentation was determined by TUNEL labeling of alternate tissue sections from PND4 mice, Mag x3.85. C) N7-mGua DNA lesion levels in the cerebellum or cerebral cortex of saline or MAM treated mice.
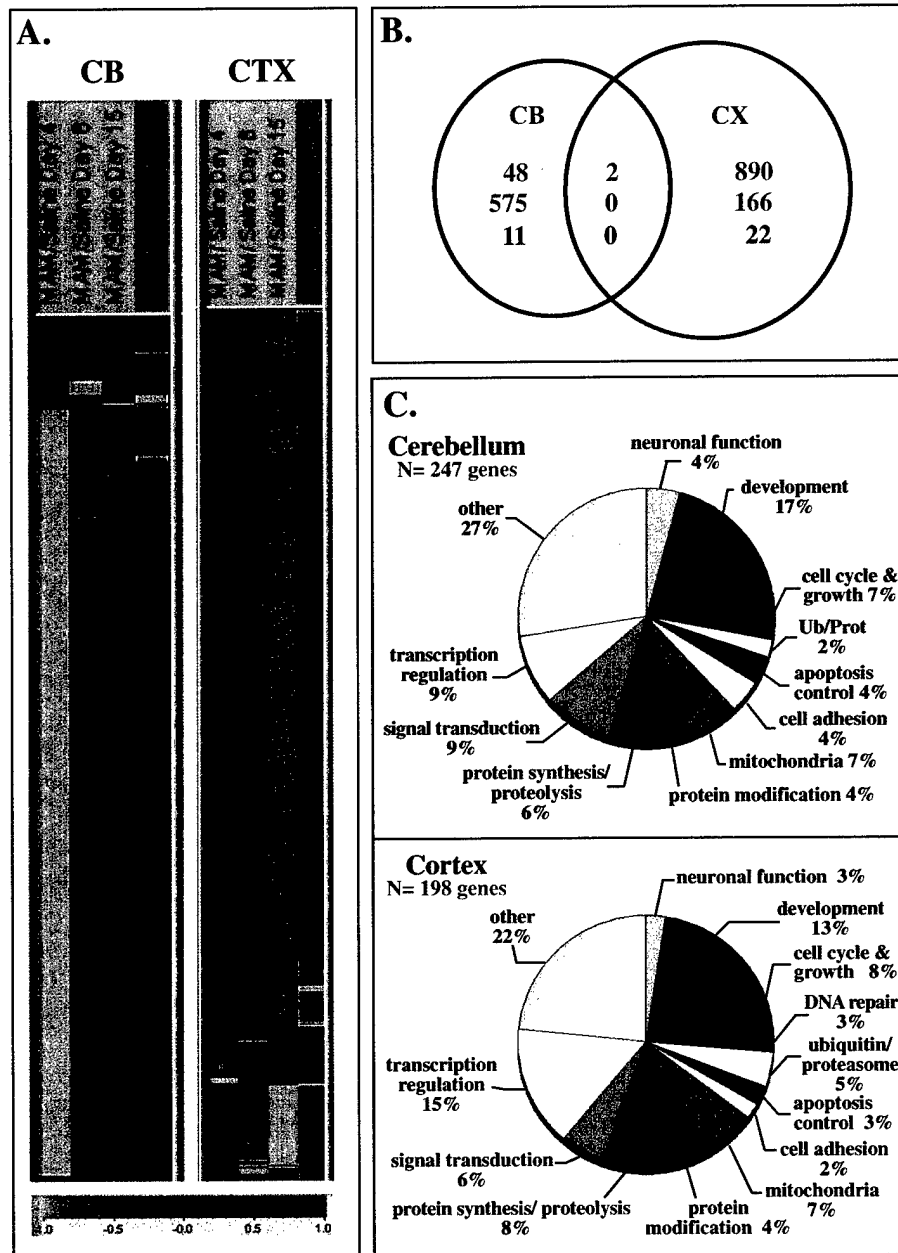
Figure 22. Effect of MAM on Global Gene Expression in the Developing Cerebellum and Cerebral Cortex of Mice. C57BL/6J mice were injected with saline or a sub-lethal dose of MAM (43 mg/kg, s.c.) at PND3 and the cerebella and cerebral cortices isolated at PND 4, 8, 15, and 22. **A.** Time course of the gene expression changes induced by MAM. All genes with log2 MAM/saline gene expression ratios greater than 1 or less than −1 for each brain region were normalized by the absolute value of the maximum fold change for the gene and grouped by hierarchical clustering using Euclidean distances. (N=636 genes for the cerebellum and 1080 genes for the cortex.). **B.** Venn diagram depicting the overlap between MAM-responsive genes in the cerebellum and cerebral cortex. Up-regulated (*red*)= numbers represent all genes with significant differences between MAM and saline treated mice and log2 (MAM/Saline)>1 at any time point; Down-regulated (*green*)= significant differences between MAM and saline treated mice and log2 (MAM/saline)<-1 at any time point, and Transient (*black*)= significant differences between MAM and saline treated mice, log2 (MAM/saline)<-1 and >1 at different time points. **C.** Functional classes of the genes influenced by MAM in the mouse cerebellum and cerebral cortex. Named genes with functional annotations in the Unigene database were categorized by broad functional class.
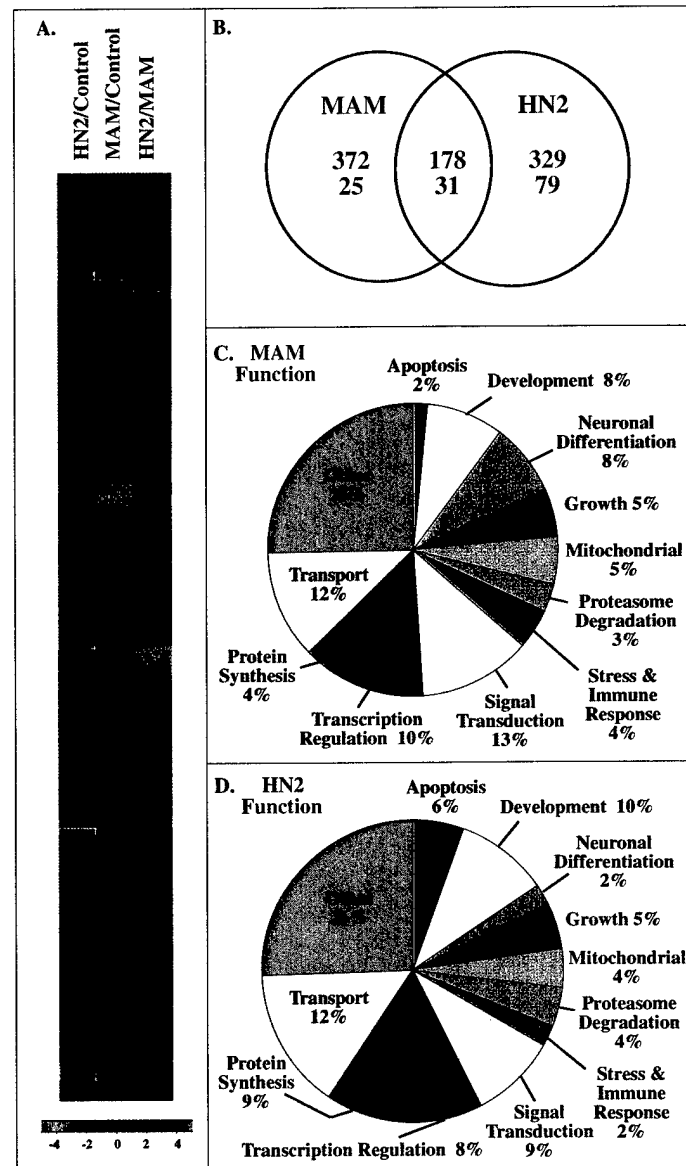
Figure 23. Effect of MAM and HN2 on Global Gene Expression in Cerebellar Neurons. Granule cell cultures prepared from the cerebellum of C57BL/6J mice were treated with 100 μM MAM or 1.0 μM HN2 for 24h. **A.** Gene expression changes induced by MAM or HN2. All genes with log2 toxin/control gene expression ratios greater than 1 or less than –1 were normalized by the absolute value of the maximum fold change for the gene and grouped by hierarchical clustering using Euclidean distances. **B.** Venn diagram depicting the overlap between MAM- and HN2-responsive genes in cultured neurons. Up-regulated (*red*)= numbers represent all genes with significant differences between MAM or HN2 and mock treated cultures and log2 (MAM or HN2/Control)>1; Down-regulated (*green*)= significant differences between MAM and mock treated cultures and log2 (MAM/saline)<-1; **C.** Functional classes of the genes influenced by MAM or HN2 in murine cerebellar neurons. Named genes with functional annotations in the Unigene database were categorized by broad functional class.

TABLE I

VIABILITY OF MICE AFTER INJECTION WITH MAM OR HN2

	Dose								
		MAM (mg/kg)			HN2 (mg/kg)				
GENOTYPE	Saline	4.3	21.5	43	2.5	5	10	20	Total *
Wild	157/157 (100)	3/3 (100)	16/26 (62)	116/215 (54)	6/6 (100)	2/17 (12)	0/4 (0)	0/2 (0)	*633*
Aag$^{-/-}$	3/3 (100)	4/4 (100)	10/17 (59)	0/5 (0)	0/1 (0)	0/1 (0)	N	0/1 (0)	*68*
Mgmt$^{-/-}$	24/24 (100)	3/3 (100)	9/15 (60)	0/6† (0)	2/2 (100)	4/4 (100)	N	N	*65*
Xpa$^{-/-}$	15/15 (100)	N	3/8 (38)	5/7 (71)	3/3 (100)	2/2 (100)	2/2 (100)	N	*43*
Mgmt$^+$	12/12 (100)	2/2 (100)	2/4 (50)	5/5 (100)	4/4 (100)	1/1 (100)	1/1 (100)	N	*29*

Values represent the number of live animals at PND 22/total number injected. ( ) % of viable mice

* Total number of animals injected for each genotype that lived to PND 22 for Years 3-5

† Animals only lived until day 15. N = none dosed